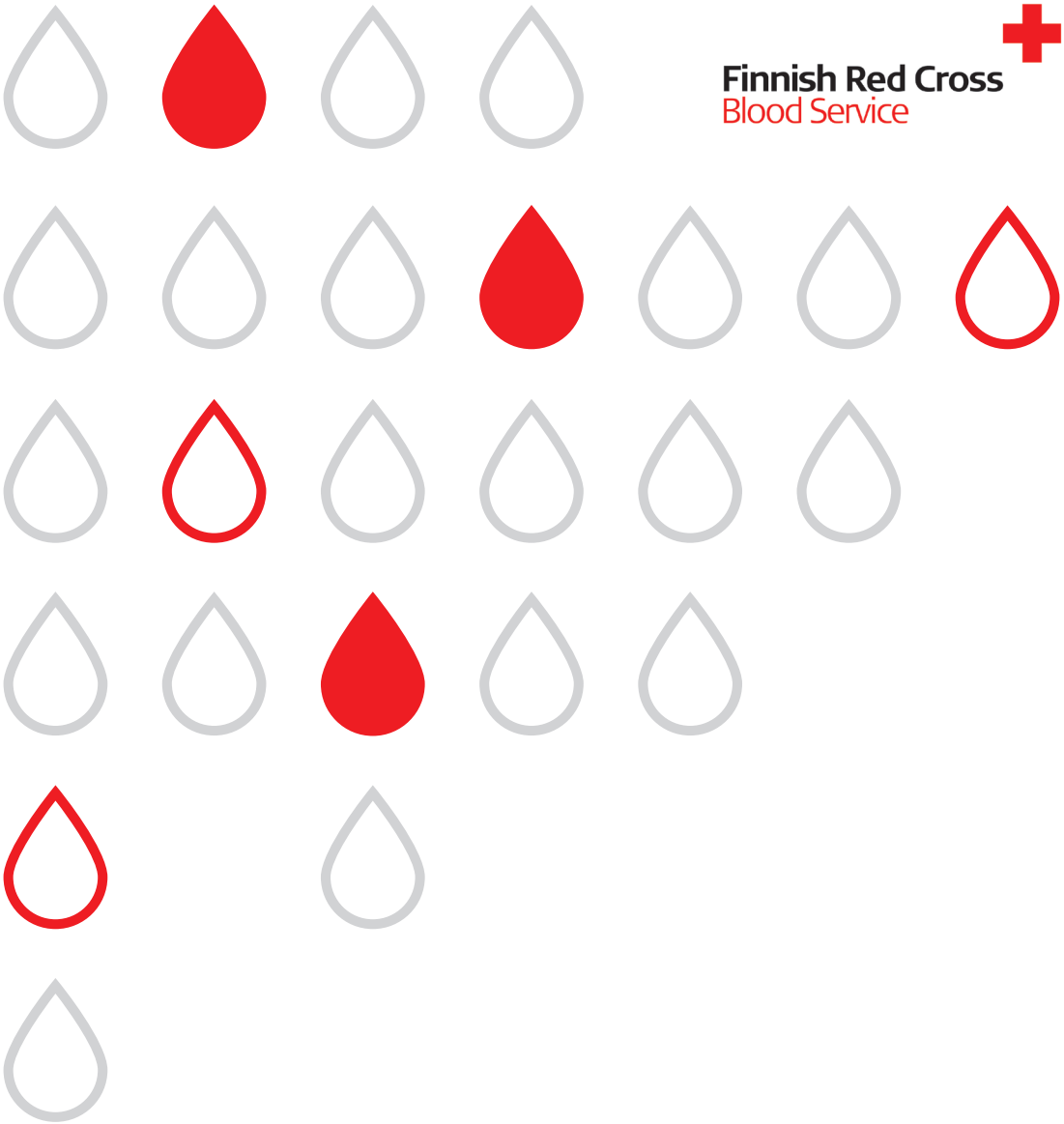

Finnish Red Cross
Blood Service

SAMI VALKONEN

Extracellular Vesicles and Nanoerythrosomes: The Hidden Pearls of Blood Products

ACADEMIC DISSERTATION, NUMBER 64

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and

Finnish Red Cross Blood Service

EXTRACELLULAR VESICLES AND NANOERYTHROSOMES: THE HIDDEN PEARLS OF BLOOD PRODUCTS

Sami Valkonen

ACADEMIC DISSERTATION

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the Faculty of Biological and Environmental Sciences of the University of Helsinki,
in Nevanlinna Auditorium of the Finnish Red Cross Blood Service,
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“All the adversity I've had in my life, all my troubles and obstacles, have strengthened me... You may not realize it when it happens, but a kick in the teeth may be the best thing in the world for you.”

-Walt Disney

To the closest ones to my heart

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ORIGINAL PUBLICATIONS

This thesis is based on the following publications that are referred to in the text by their roman numerals (Studies I-III). In addition, some unpublished results are presented.

I **Valkonen S**, van der Pol E, Böing A, Yuana Y, Yliperttula M, Nieuwland R, Laitinen S, Siljander PRM. Biological reference materials for extracellular vesicle studies. Eur J Pharm Sci 2017;98:4–16.

II **Valkonen S**, Mallas B, Impola U, Valkeajärvi A, Eronen J, Javela K, Siljander PRM, Laitinen S. Assessment of Time-Dependent Platelet Activation Using Extracellular Vesicles, CD62P Exposure, and Soluble Glycoprotein V Content of Platelet Concentrates with Two Different Platelet Additive Solutions. Transfus Med Hemotherapy 2019;46:267–75.

III **Valkonen S**[§], Holopainen M[§], Colas RA, Impola U, Dalli J, Käkelä R, Siljander PRM, Laitinen S. Lipid mediators in platelet concentrate and extracellular vesicles: Molecular mechanisms from membrane glycerophospholipids to bioactive molecules. Biochim Biophys Acta - Mol Cell Biol Lipids 2019;1864:1168–82.

[§] = equal contribution

The publications have been included in the thesis as appendix with kind permission from Elsevier (Amsterdam, Netherlands (Studies I, III)) and S. Karger AG (Basel, Switzerland (study II)).

PERSONAL CONTRIBUTION

I The author participated in the design of the study, conducted the literature review, developed and sent the questionnaire, recorded and analysed the questionnaire results, optimised the reference material production and conducted the majority of the characterisation (size and particle concentration determination, stability, flow cytometry, Nanoparticle tracking analyser (NTA) comparison), data analysis, and result interpretation. The final manuscript was written mostly by the author and it was critically revised by the co-authors.

II The author participated in the study design, performed the NTA data collection and analysis, performed the statistical analysis throughout the results and interpreted the results together with supervisors. The final manuscript was written mostly by the author, who also participated in the manuscript revision.

III The author participated in the study design, performed the NTA, phospholipid, and Western blot data collection as well as data analysis. The author interpreted the results with the co-authors, mostly wrote the final manuscript and participated in the manuscript revision.

PUBLICATIONS NOT INCLUDED IN THE THESIS

In addition to the publications included in the thesis, the author has contributed to the following publications:

Holopainen M, Colas RA, **Valkonen S**, Tigistu-Sahle F, Hyvärinen K, Mazzacuva F, Lehenkari P, Käkelä R, Dalli J, Kerkelä E, Laitinen S. Polyunsaturated fatty acids modify the extracellular vesicle membranes and increase the production of proresolving lipid mediators of human mesenchymal stromal cells. Biochim Biophys Acta - Mol Cell Biol Lipids 2019;1864:1350–62.

Siljander P, **Valkonen S**, Laitinen S, Kerkelä E. Verisolujen solunulkoiset vesikkelit. Lääketieteellinen Aikakauskirja Duodecim 2019;135:663–71.

Laurén E, Tigistu-Sahle F, **Valkonen S**, Westberg M, Valkeajärvi A, Eronen J, Siljander P, Pettilä V, Käkelä R, Laitinen S, Kerkelä E. Phospholipid composition of packed red blood cells and that of extracellular vesicles show a high resemblance and stability during storage. Biochim Biophys Acta - Mol Cell Biol Lipids 2018;1863:1–8.

Puhka M, Takatalo M, Nordberg M-E, **Valkonen S**, Nandania J, Aatonen M, Yliperttula M, Laitinen S, Velagapudi V, Mirtti T, Kallioniemi O, Rannikko A, Siljander PR-M, Af Hällström TM. Metabolomic Profiling of Extracellular Vesicles and Alternative Normalization Methods Reveal Enriched Metabolites and Strategies to Study Prostate Cancer-Related Changes. Theranostics 2017;7:3824–41.

Puhka M, Nordberg M-EE, **Valkonen S**, Rannikko A, Kallioniemi O, Siljander P, af Hällström TM. KeepEX, a simple dilution protocol for improving extracellular vesicle yields from urine. Eur J Pharm Sci 2017;98:30–9.

Aatonen M[§], **Valkonen S**[§], Böing A, Yuana Y, Nieuwland R, Siljander P. Isolation of Platelet-Derived Extracellular Vesicles. Methods Mol Biol 2017;1545:177–88.

Palviainen M, **Valkonen S**, Lindelöf A, Siljander PR-M. Solunulkoiset vesikkelit – isoja asioita pienessä paketissa. Kliinlab 2017;34:75–9.

Kerkelä E, Laitinen A, Räbinä J, **Valkonen S**, Takatalo M, Larjo A, et al. Adenosinergic Immunosuppression by Human Mesenchymal Stromal Cells Requires Co-Operation with T cells. Stem Cells 2016;34:781–90.

[§] = equal contribution.

ABBREVIATIONS

AA	Arachidonic acid
ATR	Adverse transfusion reaction
COX	Cyclooxygenase
CYP	Cytochrome p450
DLS	Dynamic Light Scattering
EM	Electron microscopy
EV	Extracellular vesicle
FA	Fatty acid
GPL	Glycerophospholipid
ISEV	International Society of Extracellular Vesicles
LOX	Lipoxygenase
LM	Lipid mediator
Mar	Maresin
NanoE	Nanoerythrosome
NTA	Nanoparticle Tracking Analysis
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Prostaglandin
PL	Phospholipase
PS	Phosphatidylserine
RBC	Red blood cell
RNA	Ribonucleic acid
Rv	Resolvin
SPM	Specialized pro-resolving lipid mediator
TF	Tissue factor
TRPS	Tunable resistive pulse sensing
Tx	Thromboxane
vWf	von Willebrand factor

ABSTRACT

Blood transfusions are aimed at increasing the cell count to a physiological level, in practice to prevent anaemia and to maintain haemostasis in the case of red blood cells and platelets, respectively. Besides participating in haemostasis, red blood cells and especially platelets are active contributors to immunology, and using the powerful arsenal of secreted factors, platelets can rapidly influence their surroundings. Many of these functions involve extracellular vesicles (EVs), lipid bilayered nanoparticles secreted by these cells. In addition to the blood cells, the transfused blood products also contain the EVs.

Currently, EVs are associated with multiple physiological and pathological conditions, but further studies to determine the exact EV-related mechanisms are needed. One major limitation in the current EV research is the lack of standardisation and comparability in the diverse technologies used to assess EVs. To directly address the repeatability and transparency of EV measurements, a biological reference material for EV studies compatible with multiple quantification and characterisation techniques was developed. This product development project involved an extensive literature search, a questionnaire sent to 50 laboratories working with EVs, the optimisation of candidate reference material production and the characterisation of the said reference material. The final reference material chosen in the study, nanoerythrosomes (NanoE), were produced by disrupting red blood cells into nanoparticles, which had similar physicochemical properties to naturally secreted red blood cell-derived EVs. The production and distribution of reference material with EV-like properties is of paramount importance as the incorporation of reference material into EV research would facilitate the inter-laboratory comparison of results and even benefit the development of technology.

As part of the assessment of temporal secretion of EVs in platelet concentrate, the potential of EVs as a novel marker for platelet activation was evaluated in ageing platelet concentrates. Measuring the EV concentration was as sensitive as a marker for platelet activation as the previously established platelet activation markers, CD62P exposure of platelet surface and the concentration of soluble glycoprotein V in the platelet concentrates, when the time-dependent activation of platelets was determined. EV concentration also revealed differential activation of platelets depending on the storage solution of the platelet concentrates.

Next, in addition to quantitative differences, compositional differences of EVs in platelet concentrates were examined by exploring one fundamental aspect of EV-mediated intercellular signalling, the lipidome. Platelet-derived EVs were shown to contain an enriched glycerophospholipid profile compared to platelets, a variety of pathway

markers of enzymatically modified fatty acids, and their bioactive forms, lipid mediators. Besides transporting phospholipids, pathway markers, and lipid mediators to recipient cells, EVs were shown to contain the required enzymatic attributes for the production of potent bioactive lipid mediators, pivotal for the active role of EVs in intercellular messaging.

To conclude, this study reports a production method for mass-producible and widely applicable reference material with EV-like properties for EV studies, a critical element of transparent and comparable EV research. Secondly, the examination of temporal secretion of platelet concentrate EVs demonstrates the value of EVs as a sensitive indicator of platelet activation. Finally, the EVs were shown to be a significant contributor to the lipid-mediated signalling of platelets.

NanoE and EVs of blood products are in a crucial position in unravelling the EV-mediated cellular functions, as they provide tools for improved, detailed research. Moreover, these nanoparticles have wide theranostic applicability, therefore they must not be considered mere *platelet dust*, as platelet-derived EVs were designated previously, but rather as the hidden pearls of blood products.

1 BACKGROUND

1.1 FROM DONATED BLOOD TO TRANSFUSABLE PRODUCTS

Transfusions of different blood components are carefully premeditated life-saving medical procedures. The main reason to transfuse red blood cells (RBCs) is to maintain sufficient oxygen supply to tissues, in practice to treat the loss of iron and RBCs, a status clinically titled anaemia [1]. Platelets or plasma are given to patients with problems in bleeding, typically caused by decreased platelet count or functionality, for instance in the case of cancer, where chemotherapy has eradicated platelets [2,3].

In Finland, three products, RBC concentrate, platelet concentrate, and plasma, can be prepared from a single unit of whole blood if the blood is processed within 24 hours of the blood donation (Fig. 1). For the donated whole blood, the first step is cell separation, where RBCs, platelets and leukocytes, and plasma are separated from each other in a bulk manner, resulting in some residual plasma contamination in cell fractions. After cell separation, the RBCs undergo leukoreduction, a process of removing the majority of residual leukocytes with filtration, and are then combined with a buffer solution containing nutrients and electrolytes to extend the functionality of RBCs [4]. After preparation, the RBC concentrates are stored at +4 °C and are transfusable for 35 days [5] counting from the blood donation.

After removal of RBCs, the remaining cell fraction called the buffy coat contains some residual plasma, most of the leukocytes, and platelets. As platelets are activated during the cell separation, buffy coats from individual donors are maintained at room temperature for a minimum of two hours before further processing to platelet concentrates to prevent the formation of platelet aggregates. The majority of the platelet concentrates in Finland are produced by combining the buffy coats of four ABO RhD-matched donors, after which the platelets in the buffy coat pool are isolated in a process also involving leukoreduction. Similarly to the RBC concentrates, platelet additive solution (PAS) is added to prevent platelet activation and to extend the platelet functionality [6]. After preparation, the platelet concentrates are stored at +25 °C under constant horizontal agitation and are transfusable for 5 days counting from the blood donation [7].

In addition to platelets and RBCs, plasma is separated and delivered to be processed into plasma-based medicinal products. The blood-cell derived extracellular vesicles (EVs) present in plasma are major contributors to the coagulation capacity of the transfused fresh frozen plasma [8], and on the other hand, they may explain the adverse transfusion reactions (ATR)

of plasma [9]. For details, see 1.2.1 The significance of red blood cell and platelet-derived extracellular vesicles.

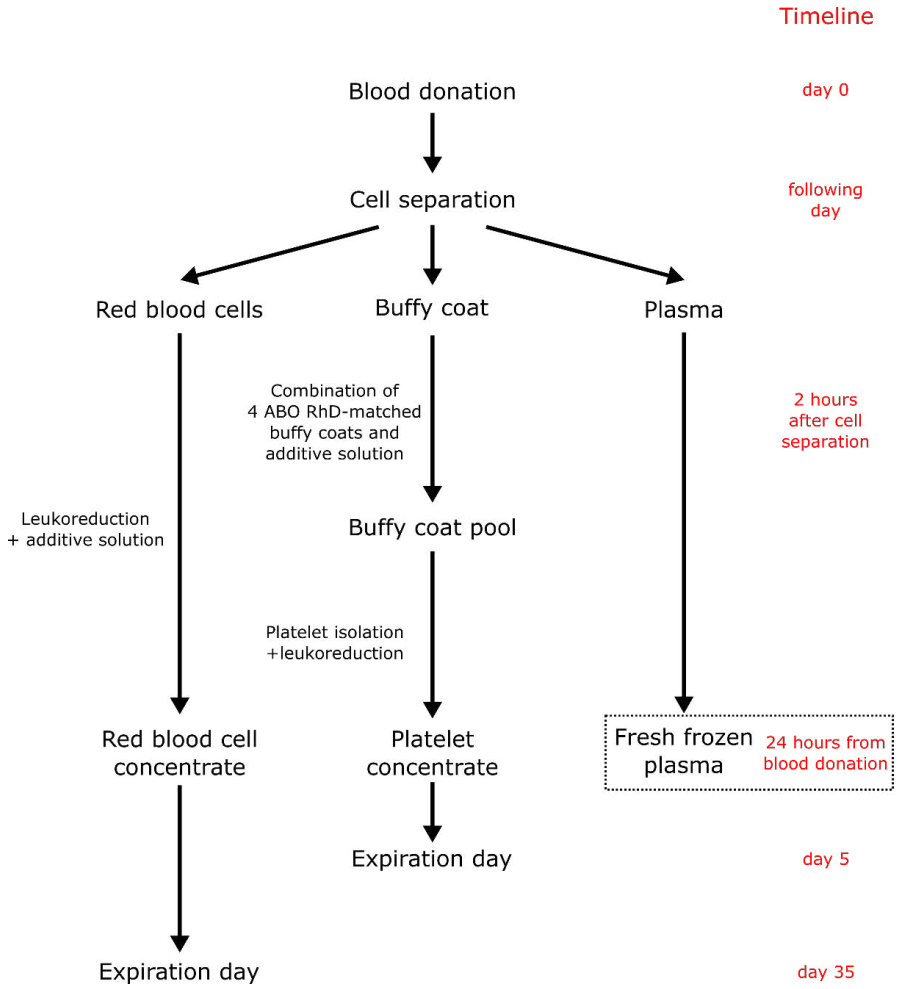

Figure 1: The simplified process of blood product preparation from whole blood at the Finnish Red Cross Blood Service.

1.1.1 RED BLOOD CELLS, DEDICATED SERVANTS OF GAS EXCHANGE

The blood circulation is an extremely efficient logistics system conveying oxygen, nutrients, waste products and heat, in addition to maintaining the intact blood circulation system and immunological defence system for the whole organism. All these functions are sustained by blood, which is a complex body fluid composed of plasma and blood cells circulating in the vasculature. Plasma accounts for 55% of the blood volume and, besides the main component, water, plasma is a rich source of lipids, proteins, ions, nutrients, and dissolved gases [10–12]. The remaining 45% of blood volume consists of blood cells, namely RBCs, platelets, and white blood cells, with a distribution of 94.2%, 5.5%, and 0.2%, respectively [10].

RBCs are anucleated cells with a diameter of 7 μm and a typical lifespan of 100-120 days in circulation [13]. The production of RBCs, erythropoiesis, is induced by erythropoietin secreted from the liver or kidney as a result of low oxygen levels in blood [14]. Erythropoiesis initiates from the multipotent hematopoietic stem cells in the bone marrow, and during the development of the RBC precursors, the cells are saturated with haemoglobin crucial for the oxygen transportation [15]. One of the final steps of the RBC maturation process involves the expulsion of cellular organelles and nucleus, explained either as an effort to maximise their haemoglobin transporting capacity [16] or to minimise their size to enable them to squeeze through small capillaries [17,18].

As structural features indicate, the central task of RBCs is the gas exchange, where RBCs bind oxygen to the iron in haemoglobin molecules and transport the oxygen to tissues. Conversely, RBCs transport haemoglobin-bound carbon dioxide from tissues to the lungs to be exhaled. Carbon dioxide is also transported dissolved in solution or buffered with water as carbonic acid, depending on factors such as oxygen partial pressure and pH [19]. Besides maintaining tissues oxygenated, the gas exchange also retains the pH of blood and tissues on the desired level [20,21].

For long, RBCs were considered to be functionally simple cells. However, with recent studies the current understanding of RBCs is changing. RBCs can influence the haemodynamics by secreting vasoactive factors and influencing the rheological properties of blood [20]. RBCs also contribute to nitric oxide metabolism and redox regulation [20], and more recently, the RBCs of human blood were also recognised as modulators of innate immunity, similarly to their counterparts in evolutionarily less developed species [22].

1.1.2 PLATELETS, MORE THAN HAEMOSTASIS

Similarly to the RBCs, platelets are produced from common myeloid progenitor cells derived from pluripotent hematopoietic stem cells. Although platelets and RBCs are both anucleated cells, the maturation processes of these cells are very different. Whereas RBC precursors actively remove their nucleus and other organelles, platelets have never contained a nucleus, as the final step of the thrombopoietin-mediated platelet production is the fragmentation of platelets from megakaryocytes (reviewed in [23]). Nevertheless, platelets have a limited capability to synthesise proteins *de novo* with their specialized translation apparatus [24,25]. Platelets have a diameter of 2-3 μm and a lifetime of up to 10 days in the human circulation [26].

In an intact vasculature, blood is actively maintained as a fluid. When the endothelial layer of vasculature is ruptured, procoagulant stimuli become exposed, initiating a sequence of cellular and enzymatic actions, platelet adhesion and aggregation and the activation of the coagulation cascade, which are the essential parts of haemostasis to minimise blood loss. Under pathological conditions, these haemostatic processes are called thrombosis. Haemostasis, the best-established functional role for platelets, consists of primary haemostasis, secondary haemostasis, and fibrinolysis. The initial responses to wounds, damaged vasculature, involve the constriction of the blood vessel mediated by the underlying smooth muscle cells, vasoconstriction [27], and the coverage of the damaged site by the incoming (circulating) platelets that adhere and subsequently aggregate, forming a temporary plug. To briefly summarise the molecular process, platelets become tethered to the immobilised von Willebrand factor (vWf) with a complex consisting of glycoprotein (GP) Ib (CD42), GPV, and GPIX located on the platelet surface. While the vWf-GPIb-V-IX interactions are not stable, platelet adhesion to the ruptured vessel site is then further stabilised by platelet interaction with the subendothelial collagen e.g., through GPIaIIa(CD49c/CD29)-collagen interactions, and GPVI-collagen mediated platelet activation, ultimately resulting in the activation of GPIIb/IIIa (or the platelet integrin CD41/CD61), which is the critical mediator of platelet aggregate i.e., the thrombus formation. The activation of platelets by collagen has several consequences: Firstly, the platelet shape changes from discoid to spherical with extensions (pseudopods), which facilitates the platelet spreading to form a monolayer plug to prevent bleeding at the damaged site. Morphological changes are paralleled with further platelet activation, especially via GPVI, resulting in the procoagulant transformation of platelets, where the phospholipid membrane is reorganised leading to the loss of the lipid asymmetry at the platelet plasma membrane and the exposure of negatively charged glycerophospholipids (GPL) phosphatidylserine (PS) and phosphatidylethanolamine (PE). This facilitates the assembly of

coagulation complexes producing thrombin. Secondly, platelet activation also results in the secretion of EVs exposing PS and PE that further promote coagulation by facilitating the thrombin production similarly to the platelet membranes [28]. Thirdly, α-granules rich in e.g., growth factors, haemostatic proteins, and adhesive proteins [29] stored in platelets fuse with the plasma membrane, causing stabilisation of the interactions between platelets already present at the wound site. As a result of α-granule fusion, platelets expose P-selectin (CD62P), a common marker of platelet activation [30]. The formation of a platelet monolayer creates the basis for the secondary haemostasis, the formation of a more stable clot, by secreting activating factors and by providing a contact surface for the additional platelets to aggregate at the wound site.

The secondary haemostasis is largely initiated by tissue factor (TF), which is mainly exposed to blood from the injury site, but EVs also contribute to the total TF activity by facilitating the production of bioactive TF [31]. Also, the role of TF in platelets is controversial, as normal platelets are not considered to express TF [32], yet TF is still found in platelets, possibly due to the fusion of monocyte or cancer EVs [33,34] or an inducible pool of TF messenger ribonucleic acid (RNA) [35], which also explains the dissemination of TF with platelet-derived EVs [36]. TF activates the more rapid extrinsic pathway of coagulation, resulting in the production of insoluble fibrin strands from fibrinogen and in the promotion of further thrombin generation that further activates platelets, thereby creating an activatory loop. Thrombin-related activation of platelets leads to the proteolytic cleaving of 69 kDa soluble part of GPV (a prerequisite for the formation of the GPIb-V-IX-complex) [37], which has also been used as a marker of platelet activation [38]. Ultimately, fibrin together with the activated platelets forms a tight clot, sealing the wound site. The final part of haemostasis, fibrinolysis, is the carefully regulated disassembly of the fibrin network mediated by plasmin derived from circulating plasminogen [39], which enables the tissue remodelling and ultimately wound healing. [40]

The given description of haemostasis above is an oversimplification of the intricate process, as for instance the role of platelets in haemostasis is more complex. Two distinct platelet populations, procoagulant and aggregatory platelets, both contributing differentially to haemostasis, exist. The formation of platelets with the procoagulant phenotype is thought to require the exposure to collagen (reviewed in [41]), but recent evidence also suggests that in trauma patients, the exposure to histones induces a platelet phenotype switch towards procoagulant platelet phenotype [42]. The common denominator for both signalling routes is the elevated cytosolic calcium concentration that will, besides the procoagulant response, activate calpain responsible of the procoagulant transformation of platelets. Procoagulant platelets are characterised by the morphological change called “ballooning”, PS exposure, inactivated

GPIIb/IIIa, and coagulation factor binding [43,44]. Whilst EVs may be formed from both types of platelets, especially the changes taking place in the procoagulant platelets are critical for EV formation, which are also thought to function as a bridge towards inflammation [42]. Haemostasis is a carefully controlled sequence of events, where the sum of activating and inhibiting signals determines platelet activation, coagulation, and fibrinolysis, not forgetting cellular interplay. Thus, opposing signals can be secreted even from the same cell type, as e.g., endothelial cells secrete TF, but also TF pathway inhibitor, a protein with extensive anticoagulant effects [45] and protein C, which inactivates the components of thrombin-producing complexes and promotes fibrinolysis [46]. However, platelets are in a pivotal role in haemostasis, as besides physically forming the clot, they are the target for the majority of the signalling molecules and actively secrete factors that promote and moderate haemostasis. Platelets produce e.g. adenosine diphosphate and thromboxane (Tx)A2, which further activate platelets to form a more stable clot and liberate coagulation factors from the secretory granules (e.g. factor V and fibrinogen), but also promote haemostasis-limiting effects by liberating e.g., TF pathway inhibitor and activating protein C [47]. Platelets also contribute to fibrinolysis in multiple ways [48–50]. To conclude, as both procoagulative and anticoagulative or profibrinolytic and anti-fibrinolytic features are present in platelets, the role of platelets in haemostasis is dependent on multiple regulating signals, a homeostatic balance, influencing several aspects of platelet functionality.

Platelets also maintain vascular integrity during inflammation [51] and facilitate the development and remodelling of the vasculature [52] and lymph system [53]. A growing amount of evidence indicates that besides being crucial mediators of haemostasis, platelets should be defined at least as an extension to the immune system, if not as actual immune cells. The active participation of platelets in immune processes is indicated by e.g., the expression of functional Toll-like receptors [54,55] capable of pathogen detection and secretion of factors contributing to antimicrobial activity, inflammation, and tissue healing [56–58]. Platelets also interact with e.g., monocytes, macrophages, T cells, neutrophils, and natural killer cells, [59–63]. Of these, inflammation is a particularly interesting aspect of platelet functionality: platelets can directly interact with leukocytes by e.g., facilitating neutrophil migration and neutrophil extracellular trap formation [62,64], but platelets also secrete cytokines that attract leukocytes and immune mediators such as complement factors and immunoglobulins [65]. Furthermore, platelets secrete bioactive lipid mediators (LM) with either an inflammation-promoting or moderating effect [66]. For details, see 1.3 Membrane lipid signalling in platelets. If platelet functionality was limited to haemostasis, having such diversity in the resources for interaction with various types of cells would not be necessary. Therefore, the view of platelets as simple contributors to

haemostasis has been shattered and replaced with an image of multifunctional cells contributing to e.g., inflammation regulation, host defence, autoimmune diseases, tumour biology, and even neurological disorders [67–72]. The highly versatile functions of platelets are relevant in blood transfusions, as the transfused blood products may cause ATRs ranging in the severity from febrile nonhaemolytic transfusion reactions to life-threatening transfusion-related acute lung injury and anaphylactic shock [73]. Compared to RBC transfusions, platelet transfusions have a higher rate of ATRs [74], underscoring the role of platelets as important mediators of immune reactions.

To better understand the platelet functions in haemostasis, immunity and inflammation, it is crucial that the highly versatile mechanisms of platelets are determined. Although the role of platelet-derived EVs as facilitators of blood coagulation is well established, the fundamental function of EVs, intercellular communication, might explain at least partly the "non-classical roles" of platelets, such as vasculature maintenance, inflammation and infections [75], since platelet-derived EVs have been shown to contain nucleic acids [76], proteins [77], and lipid signalling components [78] that enable rapid effects on surrounding cells.

1.2 EXTRACELLULAR VESICLES

The presence of EVs, "an additional thromboplastic fraction" was first demonstrated from blood plasma [79], and the procoagulant factor was later shown to originate from platelets as "platelet dust" [80]. As exemplified already by the early steps of EV research, the gradual discovery of EVs with different origins or functions has resulted in diverse nomenclature of EVs [81,82], e.g., outer membrane vesicles (EVs from Gram-negative bacteria), prostasomes (EVs from prostate gland epithelial cell), or tolerosomes (EVs from intestinal epithelial cell), to mention a few. The discrepancy in nomenclature has been addressed by the International Society of Extracellular Vesicles (ISEV, established 2011), and currently, the common term EV is suggested for "particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e., do not contain a functional nucleus", as ISEV defines EVs [83].

EVs, the fluid-filled lipid bilayer encapsulated nanosized particles, are secreted by most cells from prokaryotes to eukaryotes (extensively reviewed in [84,85]). Multiple ways to categorise EVs exist, and the most common approach is based on their formation route: EVs of eukaryotic cells are classified as exosomes secreted from cells via endosomal route [86], microvesicles budding directly from plasma membranes [87], or apoptotic bodies produced by multiple mechanisms, including direct budding and endosomal route, but having the defining characteristic of being derived from an apoptotic cell source [88].

In terms of particle size, the majority of the EV population consists of particles < 300 nm, consistent with the power-law function [89], but exosomes, microvesicles, and apoptotic bodies cannot be separated purely based on particle size. Exosomes are considered to be 30-120 nm in diameter [90], and microvesicles 50 to 1000 nm [84]. Apoptotic bodies have a wider size distribution as first demonstrated by Kerr and colleagues [91], and more recently apoptotic vesicles with a diameter of 40 nm have been reported [88].

Although there are no unique EV markers as such, because of the different biogenesis routes, the two most studied EV subpopulations, exosomes and microvesicles, have been thought to be enriched in certain molecular markers, which is considered as the basis for their classification. For example, exosomes are thought to be enriched in cell surface markers tetraspanin CD9, CD63, and CD81, and contain tumour susceptibility gene 101, whereas microvesicles contain cell organelle and surface markers from the cell of origin [84,85,90]. Despite the numerous elaborate studies reporting EV subpopulation-specific molecular markers, contradictory evidence can be readily found, demonstrating that not all exosomes contain "traditional exosome markers", and on the other hand, EVs with the molecular characteristics of exosomes have been shown to bud directly from the plasma membrane of cells [92–97]. Platelet-derived EVs as a

whole make an important exception to these generalisations, as for instance CD9 is readily expressed membrane protein in platelets [98] and CD63 is an established degranulation marker of platelets [99], resulting in their expression in both exosomes and microvesicles derived from platelets.

The reported density of the EV subpopulations vary from 1.01 to 1.30 g/cm^3 [100] and detailed analyses of EV populations with different densities have demonstrated that the density variation is due to the different molecular composition of EV populations [90,101–103]. Still, the natural EV sample cannot be separated to subpopulations in one gradient, as exosomes and microvesicles have been shown to have similar densities [95].

1.2.1 THE SIGNIFICANCE OF RED BLOOD CELL AND PLATELET-DERIVED EXTRACELLULAR VESICLES

Blood plasma is an especially rich source of EVs due to a high EV content and the presence of EVs from blood cells, along with e.g., cancer- and neural-derived EVs [104–106], as illustrated in Figure 2. The majority of plasma EVs are from RBCs and platelets, both cells contributing approximately 25% of whole plasma EVs, depending on the detection method [104,107,108]. From the historical perspective, the EVs from platelets and the RBC maturation process represent the epitome of biogenesis of microvesicles [80] and exosomes [109,110], respectively.

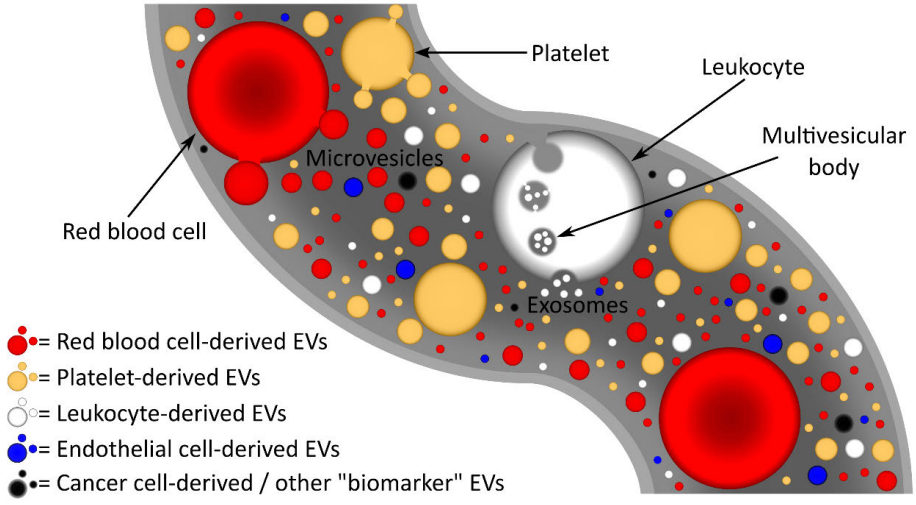

Figure 2: A schematic presentation of the extracellular vesicle (EV) diversity within blood plasma illustrating also the formation route of microvesicles and exosomes. Figure published in modified form in [111].

The diversity seen in the functional roles of RBCs and platelets can be extrapolated to their EVs: RBCs are robust cells refined to maintain the gas exchange, and in physiological context, RBC EVs have been shown to enable the optimal RBC functionality in different ways. Besides maturation from reticulocytes to mature RBCs, RBCs have been reported to increase their longevity via EV-mediated liberation of unwanted molecular content [112]. Furthermore, RBC EVs transport haemoglobin and haem between RBCs and target cells [113]. The role of RBC EVs in haemostasis is also recognised [114,115] and one of the mechanisms shown involves their exposure of von Willebrand factor [116]. In stored RBC concentrates, EVs are partially responsible for adverse proinflammatory and procoagulant reactions [117,118]. Also, RBC EVs have been shown to mediate enhanced coagulation in patients with sickle cell anaemia [119]. In malaria patients, RBC EVs enable the intercellular communication of malaria parasites and induce inflammation, which can also influence vascular function as infected RBC EVs activate endothelial cells, causing cytokine secretion attracting inflammatory cells that further activate endothelial cells [120–123].

Currently, EVs from e.g., RBCs, monocytes, neutrophils, and endothelial cells are known to facilitate different phases of the coagulation process [114,124], even though this feature was first attributed to platelet-derived EVs [80]. While the distinct role of negatively charged lipid moieties on platelets' surface and TF in coagulation has long been recognised [125], the contribution of EVs has more recently been studied in greater detail. Less than 50% of the platelet-derived EVs express PS on their outer leaflet of cell membrane [107], which is the main negatively charged lipid class responsible for the assembly of coagulation complexes. An experiment with platelet-free plasma has shown that PS-exposing EVs alone do not initiate coagulation, as also TF is required for the process, and PS-exposing EVs promote the process [126]. However, EVs can provide both factors. Through the exposure of PS and PE, which can function as binding locations for different coagulation factors [127–132], and as facilitators of the production of bioactive TF [31], platelet-derived EVs have been shown to directly induce thrombin generation [133]. TF-bearing EVs, which are detected in different physiological and pathophysiological circumstances [34,102,134], such as cancer, can initiate coagulation [33], for instance, by fusing into activated platelets. To underline the significance of EVs for haemostasis, the lack or deficiency in the composition of platelet-derived EVs has been shown to result in bleeding disorders, e.g., Scott syndrome and Castaman's syndrome [135–137], and in an experimental murine model, the removal of platelet-derived EVs resulted in reduced coagulopathy in mice with a traumatic brain injury [138]. Although plasma EVs have fibrinolytic activity, this feature has not yet been associated with RBC or platelet-derived EVs and has only been linked to leukocyte EVs [139].

Contrary to RBCs, platelets have widely established roles besides haemostasis, and EVs are shown to mediate these functions that include, but are not limited to, inflammatory or immune-system related functions [140,141], showing direct interaction with the cells of innate [142,143] and adaptive immunity [144], tissue regeneration [145], and angiogenesis [146,147], which can also facilitate cancer progression [148].

1.2.2 CARGO-BASED SIGNALLING IN EXTRACELLULAR VESICLES

While EVs can be isolated from all body fluids [85], express a vast variation in their physicochemical properties [100], and are present in various morphologies [107,149], it can be generalised that EVs are an exceptionally efficient means of transporting lipids, proteins, nucleic acids, carbohydrates, or their metabolites within the vesicles, thanks to their stable structure (Fig. 3), not forgetting the cargo carried on the external surface of EVs (reviewed in [150]). The molecular cargo includes, but is not limited to, growth factors, (anti)thrombotic signals, adhesion molecules, apoptotic signals, cytokines, and bioactive lipids (reviewed in [151]). One crucial milestone in the EV field was the discovery of EVs' capability to transport RNA, later transcribable to functional proteins in target cells [152,153]. Regarding genomic material, the RNA content of EVs has been studied more extensively than their DNA content. Extracellular RNA is considered to be either EV-associated or present as protein-complexed forms, and at least the presence of messenger RNA, long non-coding RNA, small non-coding RNA, ribosomal RNA, and microRNA has been demonstrated in EVs [154,155]. To summarise the current view, exosomes, microvesicles, and apoptotic bodies have been shown to have specific RNA and DNA profiles [94,156,157]. EVs can also transfer functional proteins to induce bursts of activity locally, for instance, in connection with lipid signalling [76,158–162]. As EVs are lipid bilayered particles, lipids are a fundamental part of EVs. Besides providing structure, however, lipids as signalling molecules have wide downstream effects and lipid signalling can be mediated via various mechanisms (see 1.3 Membrane lipid signalling in platelets).

The molecular composition of the EV surface provides the basis for the wide range of EV-mediated signalling. As indicated also by the coagulation promoting properties, one characteristic component of EVs, the PS-exposing surface, can directly interact with a variety of cell surface proteins, but alternatively it may also require intermediate proteins as bridging components for signalling to occur (reviewed in [150]). Besides PS-mediated interactions, EVs can adhere to the recipient cells with integrins, tetraspanins, proteoglycans, and lectins (reviewed in [163]), and via surface receptor-mediated contacts, EVs can act as signalling complexes and activate cells. EVs can also interact with cells by bursting

their contents to the proximity/into the recipient cell or incorporate e.g., lipids, and surface receptors into the plasma membrane of the recipient cell by fusing with the cells. Furthermore, EVs can deliver their cargo to the cytoplasmic side of cells by penetrating into the recipient cell, or, via transcytosis, travel through the cells (Fig. 3).

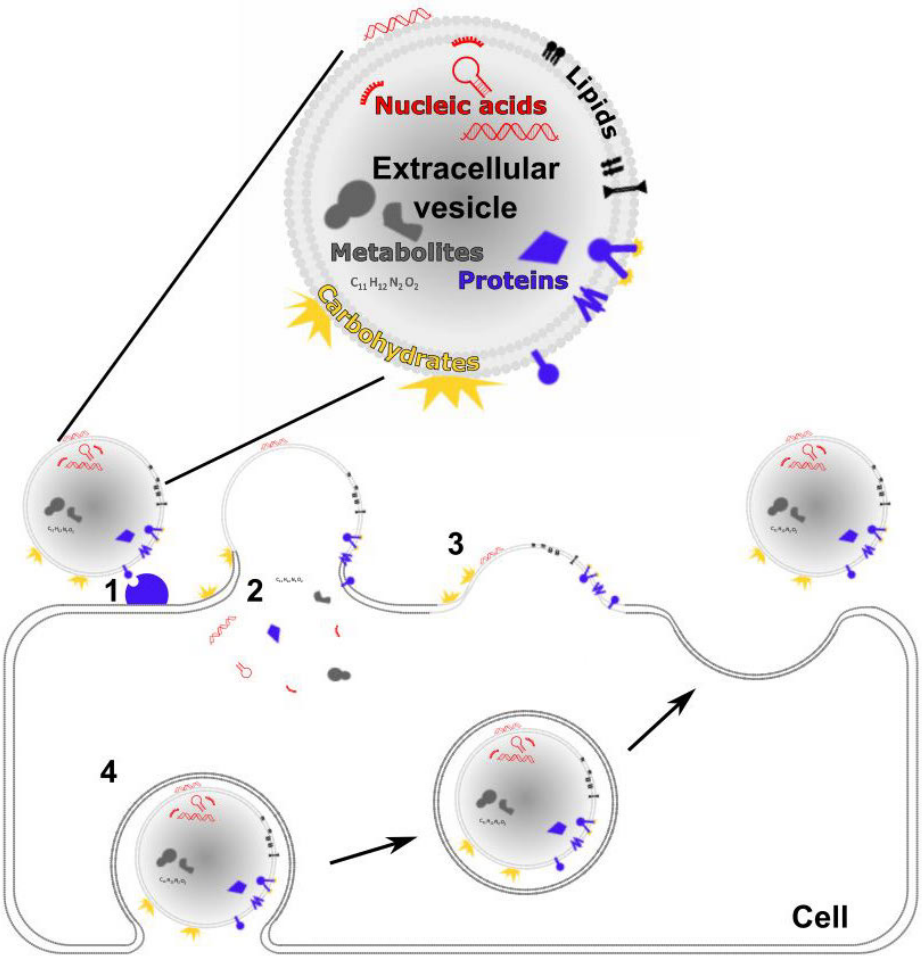

Figure 3: Schematic presentation of the molecular diversity carried by extracellular vesicles and the molecular mechanisms for their interaction with cells. 1 = receptor-mediated cell stimulation, 2 = delivery of factors to the proximity of / within cells, 3 = incorporation of molecules to the plasma membrane of recipient cell, 4 = endocytic, phagocytic, or macropinocytic uptake of extracellular vesicles into cell / transcytosis through cell. Modified from [164–166], a part of the figure published as a further modified figure in [111].

With regard to the molecular cargo of EVs, it is debatable whether the EV composition is a result of careful processing of EVs or determined by pure chance. Although the composition of EVs may be determined to some degree by pure chance, results published by various other groups suggest that because specific molecular components not required by the cells can

be expelled via EVs [109,112,167], and on the other hand, certain desired properties can be enriched in the EVs compared to the original cell [28,168], the EV composition is a carefully controlled entity. This is apparent especially in platelets, in which the composition of secreted EVs is activation-dependent: platelets secrete EVs constitutively as a part of their normal homeostasis, but when platelets are activated, their EV secretion is affected, as the EV number [169,170], composition [169–172], and procoagulant activity [173] have been shown to vary according to the conditions platelets are exposed to (Fig. 4). However, the mechanisms relating to how the EV cargo is regulated, how EVs target cells, how EVs are processed in the recipient cells and ultimately, how EVs function in health and disease still remain unsolved mysteries of the EV field [174].

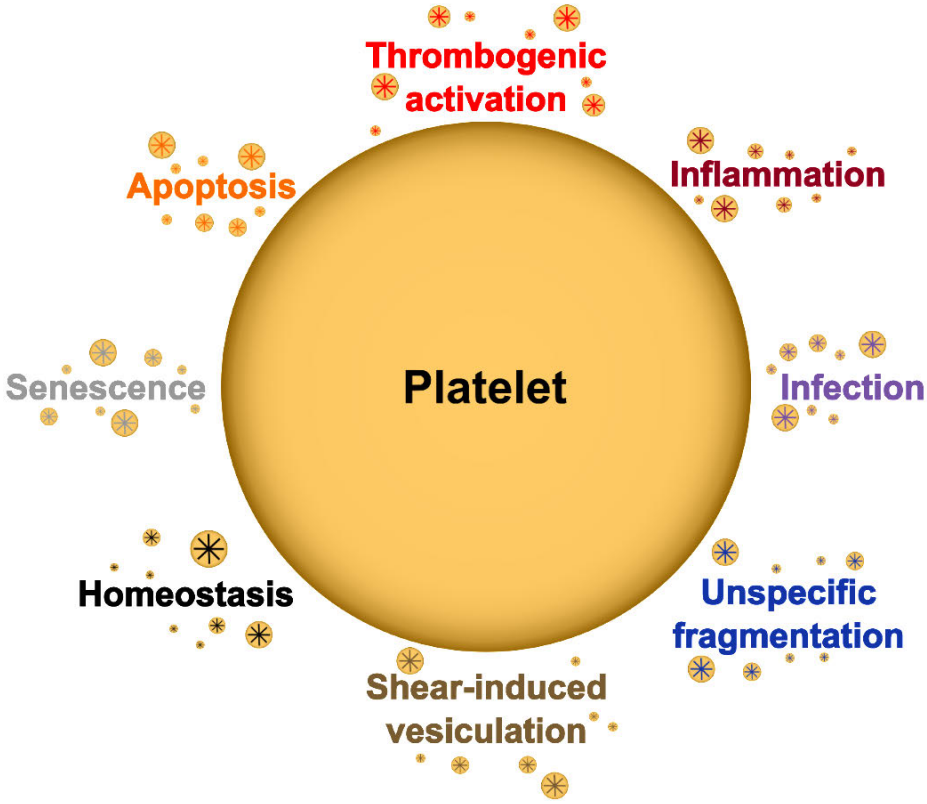

Figure 4: Agonist-dependent composition of platelet-derived extracellular vesicles.

Due to their broad molecular range, EVs are considered to be a vital part of intercellular communication an extensive spectrum of (patho)physiological functions. EVs' efficiency as molecular messengers was underlined by the discovery that EVs also enable interspecies communication: the microRNA carried in EVs from nutritional sources have been detected in the consumer and even shown to influence the

cellular responses of the consumer in animal models, as e.g., ginger EVs induce the expression of anti-inflammatory cytokine interleukin-10 in macrophages and bamboo microRNA has been detected in the breast milk of the giant panda, potentially influencing neuron development of the panda cub [175–177].

1.2.3 EXTRACELLULAR VESICLE ASSESSMENT

In EV studies, not many established facts have been defined, except for the two distinct formation routes of EVs and their remarkable capability to transfer various types of molecules. The field is rapidly developing as novel technologies emerge, which forces researchers to be alert and constantly question the current knowledge. As an example, platelets could be classified as megakaryocyte-derived EVs with the current EV definition by ISEV. EV assessment in this emerging field is highly method-dependent, and as even the current golden standards, e.g., ultracentrifugation in the EV isolation, are acknowledged not to serve their purpose properly, enormous variance in the methods to isolate, quantify, and characterise exist, influencing the reported results [178,179]. To address the variability of sample preparation and analysis methods, ISEV has released [180], and also updated [83,181], the minimal experimental requirements for EV studies to guarantee a certain level of harmonisation in the experimental settings and also to increase the transparency and comparability of data. The latest requirements are summarised in Table 1. Furthermore, ISEV actively participates in the methodological development by publishing position papers of current topics in the EV field [182–184]. In addition to these milestones, other well-cited reviews [85,185,186], international initiatives [187–189], and databases [190–192] have been compiled to guide the field and scientists starting their EV careers.

Table 1: The latest minimal information for studies of extracellular vesicles for reliable and repeatable extracellular vesicle studies. Table modified from [83].

Category	Recommendation	Examples
EV sample preparation	EV source	Quantitative description of EV source (cell number or volume of body fluid), cell culture conditions, anticoagulant
	EV yield	Quantification of EVs through lipid-, protein-, or particle amount and the ratio of these to estimate purity the of the sample
General EV characterisation	One protein from class 1, 2, and 3 If EV subpopulations are assessed, also protein from class 4 and 5	Class 1: membrane proteins (tetraspanins)
		Class 2: cytoplasmic proteins capable of binding to lipids or proteins in cell membrane (TSG101, HSP70)
		Class 3: impurities (negative control: lipoproteins, albumin, Tamm-Horsfall protein)
		Class 4: proteins (histones, cytochrome C) targeting to cellular structures like nucleus, mitochondrion, endoplasmic reticulum, Golgi apparatus, autophagosome: cytoplasm and endosomes excluded
		Class 5: soluble proteins (cytokines, growth factors) binding to EV surface receptors or demonstration of the presence of corresponding receptors
Single EV characterisation	EV characterisation using two complementary characterisation techniques	Techniques characterising single EVs, e.g., atomic force and electron microscopy
		Techniques measuring biophysical properties of EVs (size, light scattering, fluorescence, chemical composition), e.g., nanoparticle tracking analysis, flow cytometry, and Raman spectroscopy
Other characterisation	Cargo localisation	Determination of protein location within EVs, on the EV surface, or outside EVs using antibodies or with protease-, nuclease-, or detergent treatment

1.2.3.1 Isolation of extracellular vesicles

Several aspects have to be considered when choosing the EV isolation method, and perhaps the primary deciding factor regarding the EV isolation methods is the original sample, more specifically the volume and the source of the sample. Several pre-analytical aspects need to be considered especially in the case of blood samples [185,193], and getting a pure EV sample from cell culture supernatant and blood plasma requires different approaches. In addition to the amount of inherent contaminations, the starting volume may also dictate what kind of isolation methods can be used in the EV purification. Unless EVs are produced in bioreactors dedicated to producing vast amounts of EVs [194], the use of massive cell cultures consisting of multiple cell culture flasks typically results in large starting volumes for EV isolation, which may prevent the use of very specific EV isolation methods, and therefore a more general EV isolation technique is typically chosen. On the other hand, if starting material volume is limited, as it typically is in the case of e.g., clinical blood plasma samples, more specific EV isolation approaches can be applied, resulting in a very different level of purity of EVs [195], but also a lower EV yield [196]. To address the demand for EV isolation methods for samples measured in microliters, some of the most common isolation methods are also available on the microfluidics platform [197–201].

Besides the limitations set by the sample material, also the down-stream applications of EV analysis should be considered as they ultimately dictate whether the quantity or the specificity of the EV population is more important. Typically, it is not feasible to achieve both high quantity and specificity unless a substantial amount of time and money is spent on sample preparation. For a detailed molecular characterisation of EVs, the desired EV sample would ideally contain as little contaminants as possible [202]. Especially regarding mass spectrometry, the earlier sample requirements were stricter, but as mass spectrometric techniques overall have improved, less material is needed for accurate analysis, enabling molecular examination of EVs even from scarce sources [202]. If EV functionality is assessed by e.g., exposing cells to EVs generated with different methods, typically EVs are needed in larger amounts, in which case EV sample isolation may be cruder, guaranteeing that the vast majority of possible impurities are removed, while as much of the EVs and their functionality as possible is retained in the isolation processes [179].

The most commonly used EV isolation method is the centrifugation-based method, which was also the method of choice in the current study. In centrifugation-based method different EV subpopulations are typically isolated with multiple centrifugation steps using different g-values [203]. Several parameters, such as rotor type, centrifugation duration and speed, and temperature, influence the particle pelleting [203,204]. Before the

ISEV recommendations, various centrifugation protocols were applied to EV isolation [205], rendering the comparison of older results especially difficult. Differential centrifugation has also been proved to be an insufficient method for separating EV subpopulations thoroughly, to cause clumping and deformation of EVs [206,207], and also to co-isolate potential contaminants to the EV samples [203], which has to be taken into account in the consecutive experimental steps.

To provide a better separation of EV subpopulations, density gradients can be applied to ultracentrifugation [90,97,101]. Most commonly gradients are prepared with sucrose or iodixanol. As reviewed in [193], iodixanol-gradient provides better resolution and is iso-osmotic, inert, nontoxic, self-forming, and less viscous, thus requiring shorter centrifugation time. With density gradient, the sample particles are separated based on their size and density (in top-loaded gradient ultracentrifugation) or purely on density (in bottom-loaded gradient ultracentrifugation) [193,208,209]. Gradient ultracentrifugation is considered to enable EV isolation without contaminant protein aggregates and lipoproteins [210] if run for a sufficiently long time to establish equilibrium; however, one-step isolation is not sufficient to remove lipoproteins [211], as in terms of size (low-density lipoproteins, very low-density lipoproteins, and chylomicrons) and density (high-density lipoproteins), subpopulations of lipoproteins are similar to EVs [212], and furthermore, lipoproteins directly associate with EV surface [150,213].

EVs can be isolated based on the charge, density, molecular features, or size of the particles (Table 2), and along with the centrifugation-based methods, other common EV isolation methods include chromatography-, filtration-, precipitation-, or immunoaffinity-based methods. Different commercial kits, where the exact isolation mechanism is not stated, are also available for EV isolation [196,214–216].

Table 2: Different methods used to isolate extracellular vesicles.

Isolation criteria	Method	References
Charge	Chromatography	[217,218]
	Phase separation	[219]
	Precipitation	[220,221]
Density	Gradient-centrifugation	[193,222]
Molecular features	Immunoaffinity	[195]
Size	Acoustic trapping	[197,223]
	Centrifugation	[193,222]
	Chromatography	[224,225]
	Droplet evaporation	[96]
	Field-flow-fractionation	[226,227]
	Ultrafiltration	[228]

To summarise EV isolation, different techniques with their advantages and limitations exist (reviewed in detail in [179,193,228–230]), and due to the different functioning principles, the isolation technique influences the resulting EV population [215,231–233]. Finally, the more the EV sample is treated, the bigger the loss of EVs [204,234], and therefore the optimal isolation technique or combination of techniques, e.g., size-exclusion chromatography together with gradient centrifugation, that may provide a clean EV sample without a significant loss or dilution of EVs [225] is highly dependent on the starting material and the following down-stream analysis of the EV sample.

1.2.3.2 Quantification and characterisation of extracellular vesicles

Although notable variation exists in the EV isolation methods, even more variation in the results is caused by the subsequent analysis of EVs. Perhaps the most important factor in EV assessment is accurate quantification: regardless of whether the EVs are examined as a cellular response to an agonist or subjected to further analyses, accurate quantification is an absolute must. Besides meticulous quantification, the detailed characterisation of EVs cannot be ignored as it is a prerequisite to unravelling the molecular mechanisms and ultimately the biological significance of EV-mediated functions. As the EV field is rapidly evolving, different techniques are constantly being developed, in addition to the currently existing techniques being further refined to better suit the analysis of EVs.

The earliest studies demonstrated the presence of EVs with electron microscopy (EM) [79,80]. Compared to light microscopy, where studied samples are visualised using visible light and series of lenses, the use of a focused beam of accelerated electrons in EM allows the visualisation of nanoscale structures [235], including the smallest EVs. Therefore, the several different types of EM applications [236] are elementary methods even today, as accurate visualisation of EVs is required e.g., to confirm the EV isolation process [83]: EM can be used to confirm that the studied sample contains EVs and whether contaminants have been co-isolated. Additionally, EM also offers a means of characterising EVs, especially when using cryo-EM, where the native hydrated state of studied sample structures is preserved, since the sample fixing with cryo-immobilisation involves sample cooling with liquid ethane, resulting in the vitrification of water instead of ice crystal formation. When cryo-EM is combined with immunogold labelling, the size, morphology, and phenotype of EVs can be analysed in detail [107]. However, EM as a quantification method is being replaced by other techniques, mainly due to the laborious protocol involved in EM, but also due to inaccuracy, as EV concentration determination is affected by the sample preparation e.g., by the variable deposition of EVs

on to EM grids [193]. It must also be stressed that from all methods used to assess EVs, EM in particular is prone to protocol- and operator-dependent variation [237]. Initially, the cup-shaped appearance (Fig. 5) was thought to be a determining morphological feature of EVs, but nowadays the collapsed EVs are known to be artefactual, caused by the sample dehydration [186].

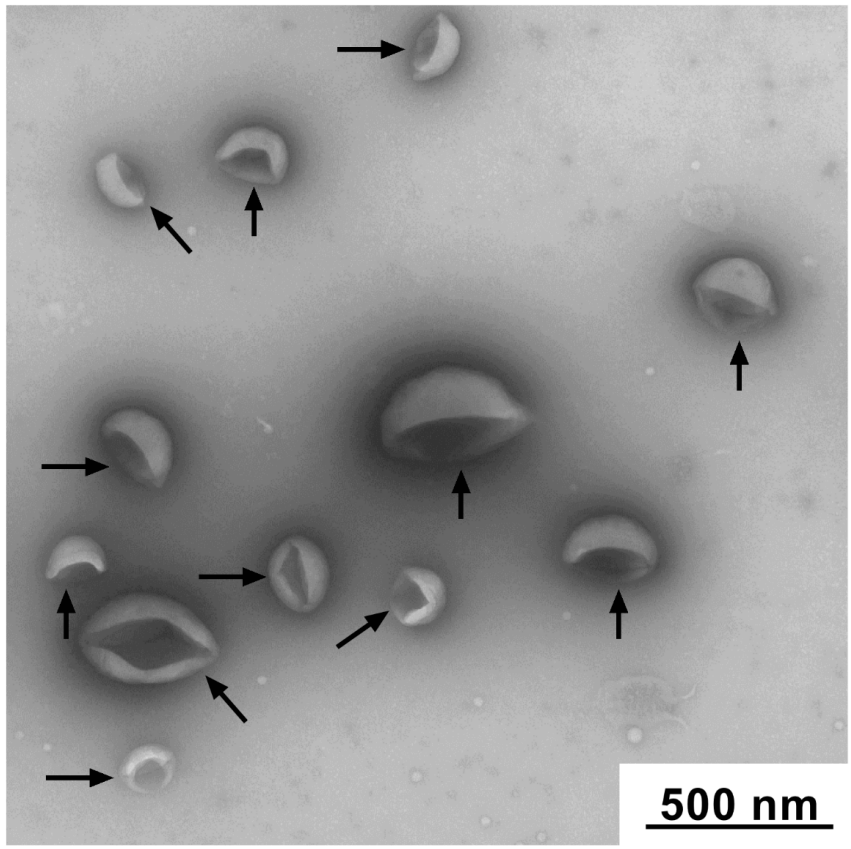

Figure 5: Electron micrograph of red blood cell-derived extracellular vesicles, where the artefactual cup-shapes are indicated with black arrows. Unpublished figure.

One of the most common methods to quantify and characterise EVs is flow cytometry, where the particle detection is based on the detection of scattered light or fluorescence. The sample particles carried by the sheath fluid are subjected to a laser beam, and the scattered light is collected with detectors located in the laser line and perpendicular to the laser line (forward scattering light and side scattered light, respectively). If the analysed structures are smaller than the wavelength of light, which is the case for majority of EV population, they produce more side scattered light. Because of this, the cellular analysis using light scatter can be generalised so that the forward scattered light corresponds to the size of particles and

the side scattered light to the structural complexity (organelles) of the sample particles. [238]

A more detailed characterisation of the molecular composition of the sample particles can be achieved by the attachment of fluorescent label to lipids, proteins, or RNA. The label is excited with a laser, and the emitted light from the label is detected by the optical system of the flow cytometer [239]. A clear advantage of flow cytometry in EV characterisation is that it is a particle-by-particle analysis as long as the EV sample is prepared in a way that prevents swarm detection, the interpretation of multiple small EVs as a single EV [240], from occurring. As the hardware and protocols develop, more accurate data of EVs can be generated using flow cytometry [241,242], resulting in better understanding of EVs and even paradigm shift, as demonstrated by the change in the plasma EV composition. Previously even 70-90% [243] of the blood EVs were thought to be platelet-derived purely because the detection limits of first-generation flow cytometers tailored for cell analysis enabled only partial detection of the EV population [244]. However, with current technologies, the percentage has been shown to be significantly lower, approximately 25-40%, depending on the detection method [104,107,108]. Besides developing more sensitive flow cytometers, also completely new types of flow cytometer methods have emerged, combining the advantages of flow cytometer sample preparation and assessed particle number with the imaging of microscopy that enables, for instance, more detailed investigation of particle uptake [245–247]. However, as mentioned previously, any labelling-based detection of EVs is cumbersome due to the heterogeneity of EVs, physical restrictions regarding antigen expression of EVs [108], and micelle or aggregate formation in the case of lipid dyes [242,248].

As a clear demand for label-free methods to examine EVs existed, dynamic light scattering (DLS) was applied to EV quantification and sizing. With DLS, however, the accurate sizing of sample particles can be conducted only with samples consisting of monodisperse particles [249], and as EV samples are polydisperse, DLS was found to be a suboptimal method to analyse natural EV population, and therefore nanoparticle tracking analysis (NTA) has largely replaced DLS.

NTA is currently offered commercially by a few companies [250], and the technique is based on sample particle illumination with a laser in a measuring chamber, where the number of visible particles in a determined dilution is used to calculate the particle concentration in the sample [251]. In NTA, the random movement of particles, called Brownian motion, is used to deduce the size of the sample particles, as based on Stokes-Einstein equation, the Brownian motion and particle size correlate inversely [252]. An interesting publication, however, points out, that the protein content of EVs might hinder the particle mobility [253]. Furthermore, various sources of variability (e.g., type of camera, depth of laser beam, and optical alignment) regarding NTA have been identified [254], and one clear

limitation of NTA is the rather high detection limit with regard to EVs, approximately 70 nm [89].

Another method to quantify and size nanoparticles, Tunable resistive pulse sensing (TRPS), is also offered commercially by two companies [255]. The method is based on Coulter principle, a widely used cell quantification technique where sample particles are enumerated and their size is determined by detecting a change in the electric current, which is caused by the sample particles that move from one electrolyte-filled chamber to another via microchannels or pores of the setup. The number of blocks is related to the particles passing the pores and in a known volume that can be translated into particle concentration. Whereas the concentration is determined based on the frequency of the current alterations, the magnitude of the resistance in predefined current relates to the size of the particle passing the pore [256]. The limitation of TRPS is related to the simplicity of the technique: For the particles to be detected, they have to pass the pores of the measurement setup. However, sample particle charge impact the electrophoretic mobility of the particles and pore passing [257]. Additionally, the pores come in predefined sizes, which might prevent quantification and characterisation of samples with highly polydisperse particles [258]. Upon aging the nanopores also become vulnerable to stretching. DLS, NTA, and TRPS are designed for the same task, mainly to quantify and determine the size distribution of the sample particles, and all three techniques, especially NTA, are widely used in EV studies.

The molecular composition of EVs can be examined in detail with mass spectrometry, where the sample is ionised and typically also fragmented, after which the mass-to-charge ratios of fragments are determined and used to identify the fragments [259]. The various applications of mass spectrometry (reviewed in [259,260]) have been widely applied to examine especially the proteome of EVs [95,261,262], but the interest and the number of publications regarding EV lipidome [262–264] and metabolome [168,194] are constantly growing as EV-mediated signalling is not limited only to the protein-based interactions.

In addition to the described techniques, EVs can be characterised using different biochemical analyses, electrochemical sensing, immunoassays, Raman spectroscopy, and small-angle X-ray scattering. Techniques that can quantify and characterise EVs also include atomic force microscopy and interferometric imaging (Table 3). Especially interferometric imaging of single particles is an interesting novel development, where the number, size, and phenotype of studied EV populations captured to silicon substrate using immobilised antibodies can be analysed using digital optical detection capable of detecting particles with the diameter of 50 nm [265]. Similarly to isolation methods, EVs can also be characterised on microfluidics platforms [199,266,267].

As a summary, several methods to quantify and characterise EVs exist, but the techniques have notable variation, as some are capable of

"ensemble" analyses (e.g., mass spectrometry, Raman spectroscopy, DLS) whereas others provide "particle-by-particle" analyses (e.g., microscope-based techniques, flow cytometry, TRPS). Another aspect is the type of results the techniques give, which could be classified as "direct measurements" (surface marker presence in flow cytometry or diameter with TRPS) or "indirect estimates" (diameter estimation in NTA).

Table 3: Techniques used to quantify and characterise extracellular vesicles. Table modified from [268]. * = truly quantifying techniques applied for extracellular vesicle quantification.

	Technique	Characterisation	References
Characterising methods	Biochemical analyses	Composition	[268]
	Electrochemical sensing	Various, mainly cargo or surface markers	[269–272]
	Immunoassays	Composition	[273,274]
	Mass spectrometry	Composition	[202,275]
	Raman spectroscopy	Composition	[276]
	Small angle X-ray scattering	Size, structural information	[257]
Particle enumerating and characterising methods	Atomic force microscopy*	Molecule interaction, morphology, size, surface marker	[277–279]
	Dynamic light scattering*	Size	[249]
	Electron microscopy	Composition, morphology, size	[236,280]
	Flow cytometry*	Composition, size	[281]
	Interferometric imaging*	Size, surface markers	[265]
	Nanoparticle tracking analysis*	Size, surface marker, zeta potential	[251,254,282]
	Tunable resistive pulse sensing*	Size, zeta potential	[257,258,282]

1.2.4 BEYOND BIOLOGY, TOWARD APPLICATIONS AND COMMERCIAL VALUE

The active research to understand the biological significance of EVs, need-based development and standardisation of EV-based methodology and protocols, and substantial efforts to generate EV-based applications have resulted in an exponential growth of EV-related publications in recent years (Fig. 6). The potential of EVs as tools in therapeutics and diagnostics,

with a combined term theranostics [182,283–285], has led to an estimation that the market value of EV-related products will reach 2.28 billion USD by 2030 with the compound annual growth rate of 18.8% [286].

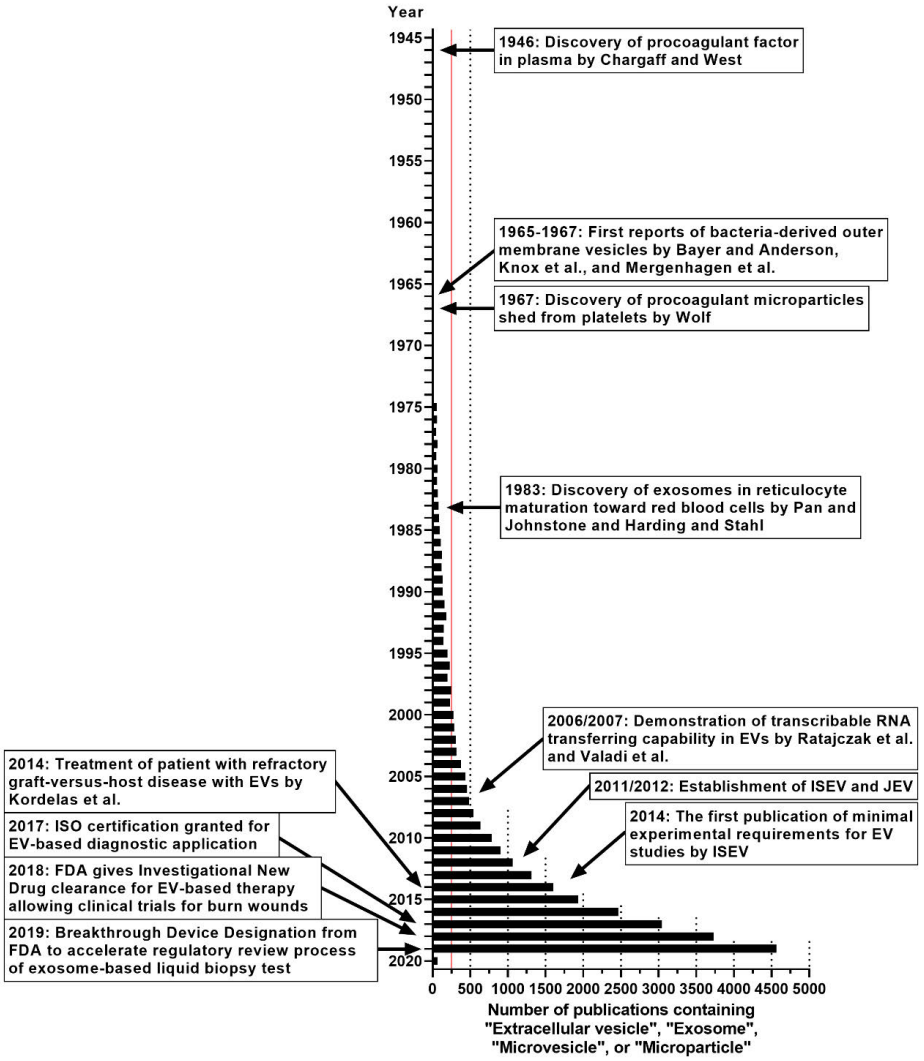

Figure 6: Timeline with the number of extracellular vesicle (EV) publications and important events of EV field. For further information, see references [79,80,290–294,109,110,152,153,180,287–289]. Amount of publications confirmed from PubMed on 3.12.2019. FDA = Food and Drug Administration; ISEV = International Society of Extracellular Vesicles; ISO = International Organisation of Standardisation; JEV = Journal of Extracellular Vesicles.

Due to their principal role as intercellular messengers and particles that are taken up by cells, EVs are also considered to be excellent drug carriers. EVs derived from e.g., cell culture supernatants, bovine milk, plants, and microbes have been suggested for drug delivery systems [295–297], but especially RBC EVs have been appraised as mass-producible,

safe, and stable candidate for drug delivery vehicle production [298,299]. EVs can also function as natural therapeutics due to their specific molecular assortment [182]: Ground-breaking work of Kordelas and colleagues has demonstrated the value of EVs in the treatment of graft-versus-host disease [291], and in 2018 Food and Drug Administration permitted the initiation of clinical trials of burn wound patient treatment with EVs [293].

EVs are acknowledged as valuable biomarkers, potentially enabling earlier diagnosis of e.g., cancer [283,300], and neurological diseases [106] through cancer cell-derived EVs and neural EVs, respectively. Also, the Food and Drug Administration (US) has recognised the biomarker value of EVs, as attested to by the granting of the accelerated review process for an EV-based diagnostic application [294]. Increase in the plasma EV concentration, and especially platelet-derived EVs, have also been suggested to serve as a biomarker for trauma, atherothrombotic disorders, autoimmune diseases, and cancer [126,244]. As the composition of platelet-derived EVs is activation-dependent [169–172], the careful characterisation of platelet-derived EVs could result in disease-specific molecular biomarkers, which would be a more reliable and repeatable basis for diagnosis than EV quantification.

The first set of minimal experimental requirements in EV studies were published only in 2014 [180], three years after ISEV was established, and although the lack of standardisation of EV research is a notable issue, standardised and certified EV-based diagnostic applications already exist [292]. Another aspect concerning especially blood plasma-derived EV application in therapeutics is the legislative classification of EVs: while Food and Drug Administration considers EVs as biological medicinal products, are they regarded as blood products (or as cells and tissues, if isolated from other sources), advanced therapy medicinal products, "others", or should they be classified in the yet-to-be-established category of EVs [182]? European Medicines Agency has defined EVs from gene-modified cells as gene therapy, in practice as advanced therapy medicinal product, whereas mesenchymal stem cell-derived EVs are not considered as advanced therapy medicinal product [301], indicating that the method of action is the defining aspect in EV classification.

1.3 MEMBRANE LIPID SIGNALLING IN PLATELETS

Membranes are a fundamental part of every organism since defined functional entities such as cells, EVs, and cellular organelles are membrane-covered structures [302]. The plasma membrane of the mammalian cell consists of phospholipids, cholesterol, and glycolipids, and the assembly of lipid bilayer, the central element of plasma membranes, is based on hydrophobic interactions: like all lipids, e.g., phospholipids consist of nonpolar (hydrophobic) hydrocarbons, typically carbon chain attached to a carboxylic acid or fatty acids (FA), and a polar (hydrophilic) part, phosphate in the case of phospholipids. The main component of plasma membranes in mammalian cells, phospholipids, can be categorised as GPLs and sphingolipids, where FAs are linked to (diacyl)glycerol or to ceramide, respectively. In addition to the FAs, glycerol or ceramide, and phosphate group, phospholipids contain a varying head group, the basis for the further classification of phospholipids (Fig. 7A). The main head groups of phospholipids are choline, ethanolamine, serine, and myo-inositol, which correspond in GPLs to head groups of phosphatidylcholine (PC), PE, PS, and phosphatidylinositol (PI), respectively (Fig. 7B). In mammalian cells, especially PE but also PC can additionally be present as plasmalogens, where ether bond to the alkenyl group is present in the *sn*-1 position [303]. In contrast to phospholipids, glycolipids consist of carbohydrates attached directly to the glycerolipids or sphingolipids, and depending on their nonpolar section they are classified as glycoglycerolipids or glycosphingolipids, respectively. Glycosphingolipids can also be further classified based on their carbohydrate structures. Glycolipids account for 2% of mammalian cell plasma membranes and are located exclusively at the extracellular surface of plasma membranes, mainly at lipid rafts with cholesterol. [304–306]

Besides providing structure by forming a physical barrier separating the cellular contents from their surroundings, lipids offer an evolutionally conserved [307], efficient way to preserve energy, due to their high reduction state, which can be exploited to power cellular functions when required [308]. It is well established that the dietary fats influence the cellular lipid membrane composition and functionality [309] and furthermore, we have shown that supplementing FAs to human mesenchymal stromal cells also influences the lipid composition of EVs [310].

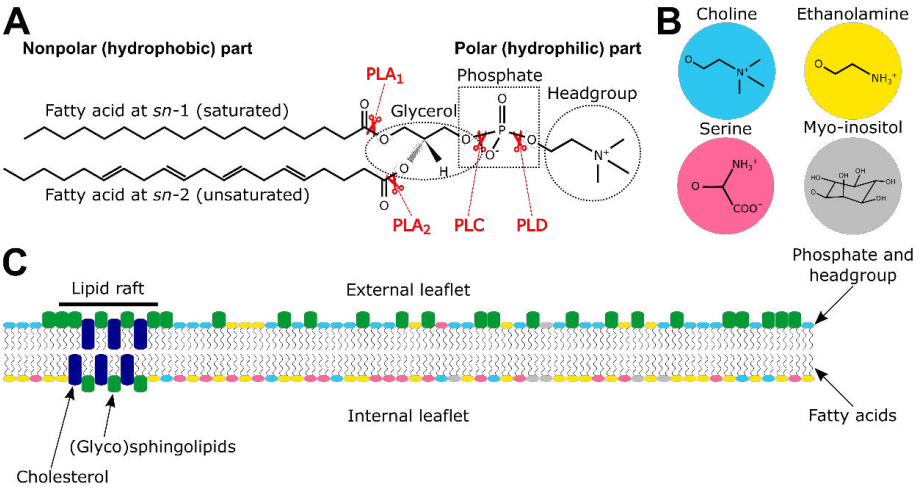

Figure 7: [A] General structure of glycerophospholipids where the cleavage sites of phospholipase (PL) enzymes are indicated with red, [B] molecular structure of most common glycerophospholipid head groups, and [C] the asymmetric lipid membrane of red blood cells. Image modified from [260,311].

Moreover, lipids serve also a vital role in signalling by participating in signalling processes via multiple distinct mechanisms. The complexity of lipid-mediated signalling can be observed even on the molecular interactions on plasma membrane: lipids provide a stable yet dynamic environment for other molecules to interact, as demonstrated by the presence of carbohydrates, nucleic acids, and proteins on plasma membrane [312–314] and the presence of e.g., immunoglobulins, coagulation factors, cytokines, and lipoproteins on EV surface [150]. A level of complexity is added to this type of participation by lipid rafts consisting of (glyco)sphingolipids and cholesterol. These distinctive structural microdomains of plasma membrane have been shown to mediate multiple different signalling processes, including immunoglobin E signalling and T-cell receptor signalling (reviewed in [315]).

Lipids also participate in signalling directly. The asymmetric lipid composition of plasma membrane (Fig. 7C) is a carefully controlled entity, and the aminophospholipid translocase and phospholipid scramblase-mediated loss of membrane asymmetry [316], manifested as the exposure of PE and especially PS on the extracellular side of cell membrane, is a recognised marker of apoptosis, whereas, for platelets, it is a marker of cellular activation [317] and a crucial part of haemostasis, as the negative charge facilitates the production of thrombin [40]. As a curiosity, the RBC membrane is one of the best-characterised membranes [318] due to the functional and structural simplicity of RBCs. In platelets, the lipid asymmetry is not yet determined to a similar level of detail.

Lipid signalling can also involve only a certain part of the lipid molecule, as secondary messengers can be produced either from the polar head group or nonpolar hydrocarbon tails via enzymatic modification [319]

or even with spontaneous oxidation processes [320]. Perhaps the best-known example of the head group-mediated signalling is the PI-signalling (reviewed in [321]), which is also an important aspect of exosome secretion: PI-signalling is a known regulator of membrane dynamics and vesicular transport, and preventing PI-signalling reduces the autophagic degradation of multivesicular bodies, possibly due to impaired membrane fusion, which results in promoted secretion of exosomes [322].

Recently, signalling mechanisms involving bioactive metabolites of GPL-derived FAs, termed as LMs, have attracted increasing interest due to their pivotal role as powerful regulators of inflammation. LMs can promote inflammation or have pro-resolving effect, which actively dampens the inflammation. Pro-resolving function is a distinct mechanism from anti-inflammatory effect: as an example, blocking the neutrophil recruitment would be an anti-inflammatory measure, whereas promoting macrophage phagocytosis to remove microbes is a pro-resolving measure. The functional nature of LMs provides a way to categorise LMs, as they are titled proinflammatory LMs (e.g., TxA_2) or specialized pro-resolving mediators (SPMs, e.g., Resolvins (Rv), Protectins, and Maresins (MaR)), and some LMs, such as Prostaglandin (PG)E_2, exhibit dual functions [323]. Structurally LMs are divided to eicosanoids and docosanoids according to the length of the carbon chain (20 or 22 carbons, respectively) of the original FA.

Analogously to EVs, only recent technological advancement has enabled an accurate characterisation and assessment of LMs that are bioactive in pico to nanomolar concentrations [324]. The novelty of this emerging field of studies is underlined by the recent discovery of novel classes of lipid mediators [325]. The enzymatic processing of FAs involves a sequence of enzymatic steps, initiating from the FA cleavage from the GPL by phospholipases (PL) (Fig. 7A). Both the cleaved FA and the participating enzymes, which modify the intermediate products (COX, lipoxygenases (LOX), and members of cytochrome P450 (CYP) family) influence the functions of the produced LM. The diversity is best exemplified with the metabolism of arachidonic acid (AA), an FA with a carbon chain consisting of 20 carbons and 4 double bonds starting from 6^{th} carbon from the omega end, which can serve as a precursor for proinflammatory LMs, SPMs, and dual-function LMs (Fig. 8).

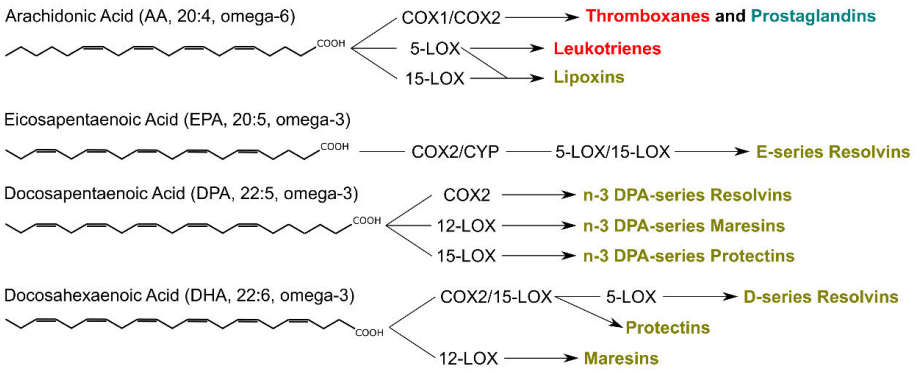

Figure 8: Simplified representation of lipid mediator production from fatty acids. Proinflammatory lipid mediators indicated with red, specialised pro-resolving lipid mediators with green, and dual function lipid mediators with teal. For detailed mechanisms of lipid mediator production, see [326–330]. COX = Cyclooxygenase, CYP = Cytochrome p450, LOX = Lipoxygenase.

LMs have substantial significance in the inflammation, as they have been shown to directly exert their proinflammatory or proresolution influence on the cells in an inflammatory milieu [331–338]. Currently, the role of LMs in several chronic inflammatory diseases, such as rheumatoid arthritis, asthma, and atherosclerosis [339] and, on the other hand, the survival-promoting effect of SPMs in e.g., lung injury and burn wound sepsis with murine model [340] has been well established.

The diversity of LM signalling can also be seen in platelet function as both proinflammatory LMs and SPMs directly influence platelet functionality. The most abundant interaction between LMs and platelets is the activation-dependent production of AA-derivatives, capable of exerting pro- and antithrombotic influence on platelets [341]. For instance, TxA_2 is a vasoconstrictor and platelet activator [342] with a half-life of 30 seconds [343]. However, the same AA platelets can also produce LMs with opposing effects, such as PGD_2 and PGE_2. As already stated, the novel interesting roles of platelets indicate their active participation in immune modulation. Besides propagating platelets' functions, for instance, the secreted TxA_2 also activates monocytes and induces T-cell differentiation, to mention a few examples of the influences they exert on the surrounding cellular milieu. The wide range of effects of TxA_2 also partially explain why aspirin, a commonly used inhibitor of COX1 producing TxA_2 [344], is considered as a beneficial treatment in inflammatory and cancer context [345]. Aspirin has also been shown to induce SPM production [346].

Although LM-mediated signalling has not been as extensively studied in RBCs as in platelets, curiously a decreased polyunsaturated FA content of RBCs has been speculated to associate with Alzheimer's disease [347]. The increased oxidative stress of RBC concentrates, which is related to storage time, has also been shown to result in the non-enzymatic generation of LMs that influence platelet functionality [348].

2 AIMS OF THE STUDY

The lack of consistency in EV analysis technologies hinders the comparison of EV studies, preventing a detailed understanding of the mechanistic aspects of EV functionality, a prerequisite for EV-based theranostic applications. To fully unravel the potential of EVs, the conducted research should be transparent and traceable, achievable, for instance, with the use of widely applied standards. Recognising the significance of EV components is crucial, especially with regard to cellular therapy products, such as platelet and RBC concentrates, where EVs accumulate in a closed system. Besides being indicators of the cellular status and therefore having the potential to act as quality control measures, firm evidence exists that EVs are major contributors in health and disease. EV-mediated effects also involve lipid signalling cascades, which are considered as an especially potent immunomodulatory signalling route, but the details of how EVs contribute to lipid signalling are still poorly understood.

The overall aim of this study was to improve the comparability of EV measurements and to analyse in detail the temporal secretion of EVs and accumulation of potent lipid signalling components to EVs in a platelet concentrate.

The specific aims of this thesis were

- To develop biological reference material compatible with EV quantification and characterisation methods based on different techniques (Study I)
- To evaluate the relationship of platelet activation and platelet-derived EVs together with sGPV production and CD62P exposure of platelets (Study II)
- To assess the time-dependent lipidome alteration of platelet concentrate-derived EVs (Study III)
- To examine the molecular mechanisms of bioactive lipid mediator production in platelet concentrate-derived EVs (Study III)

3 MATERIALS AND METHODS

The materials and experimental methods used in this study have been described in detail in the original publications (Table 4) and hence only brief descriptions of the methods are provided here with references to the original publications.

Table 4: Materials and methods used in the thesis project.

Material or method	Study
Blood products	I-III
Extracellular vesicle isolation	I-III
Reference material production	I
Reference material characterisation	I
Platelet activation assessment	II
Membrane lipid and fatty acid analysis	III
Lipid mediator and pathway marker analysis	III
Flow cytometry	I-III
Nanoparticle tracking analysis	I-III
Western blot	III
Statistical analysis	I-III

3.1 BLOOD PRODUCTS

The source for nanoparticles in all publications were blood products of the Finnish Red Cross Blood Service or Sanquin. In Study I, the nanoerythrosomes (NanoE) were prepared from RBCs isolated from RBC concentrates and the natural RBC EV population was isolated from the respective RBC concentrates produced by Finnish Red Cross Blood Service and Sanquin. In Studies II and III, the platelet concentrates produced by Finnish Red Cross Blood Service consisted of the platelet pools derived from 4 buffy coats of ABO RhD-matched donors. In Study II, the time-dependent platelet activation and the effect of storage conditions on the platelets were assessed by examining platelet concentrates with different PAS, the main difference being that PAS-E contains potassium, chloride, and phosphate, whereas PAS-B does not (Table 5). In Study III, the assessment of lipid signalling in platelets was conducted with platelet concentrates with PAS-E.

Table 5: Compositional differences of platelet additive solution (PAS)-B and PAS-E. Table modified from [6].

Component (mM)	PAS-B	PAS-E
Sodium chloride	116	69
Sodium acetate	30	30
Sodium citrate	10	10
Potassium chloride	-	5
Magnesium chloride	-	1.5
Phosphate	-	26

3.2 EXTRACELLULAR VESICLE ISOLATION

In all publications, EV isolation was performed by differential centrifugation. The isolation of EVs from RBC concentrates (Study I) involved a process of concentrate dilution and several low-speed centrifugations together with high-speed centrifugation to isolate the final EV population.

When the EVs were isolated from platelet concentrate (Studies II and III), a similar protocol with concentrate dilution and low-speed centrifugations to remove cellular contamination and high-speed ultracentrifugation to isolate the final EV population was applied, but the protocol additionally involved the inhibition of platelet activation with Apyrase and Anticoagulant Citrate Dextrose before the centrifugations.

3.3 REFERENCE MATERIAL PRODUCTION

Before the actual production of the reference material, a literature search of available nanoparticles for candidates for the reference material was conducted. A questionnaire (Appendix IV) was sent to 50 laboratories working with EVs, including questions about the desired properties of the reference material and, for instance, the currently used techniques.

To produce reference material, RBCs were broken down to nanosized particles titled NanoE, and three protocols, freeze-thaw cycles, N_2 bomb, and ultrasonication of RBCs were tested in the production of NanoEs. The presumed membrane fragments were washed and sealed with incubation at 37 °C.

3.4 REFERENCE MATERIAL CHARACTERISATION

To assess the efficacy of different methods to break down RBCs, the resulting fragments were fixed, negative stained using uranyl acetate, and imaged with transmission electron microscopy. To determine the size distribution and stability of NanoEs in storage, they were subjected to NTA

measurement. To compare NanoEs to naturally produced RBC EVs, their refractive indices were determined, and both CD235a and Di-8-ANNEPS positivity was compared using flow cytometry and protein composition was analysed using silver-stained SDS-PAGE gels.

3.5 PLATELET ACTIVATION ASSESSMENT

The platelet activation was assessed using the CD62P exposure of the platelets determined with flow cytometry and the production of soluble GPV with a commercial kit, while NTA was used to assess the EV production of the platelets and to determine the EV size.

3.6 MEMBRANE LIPID AND FATTY ACID ANALYSIS

The FAs of the platelet concentrate were quantified as fatty acid methyl esters using gas chromatography. The identification and quantification of fatty acid methyl esters were confirmed using authentic standard mixtures that were run together with the samples.

To determine the phospholipid profile alterations of the platelet concentrates, platelets, and EVs, the different sample types and time points were all isolated from the same 5 platelet concentrates. The lipids were extracted and infused into the electrospray ionisation source of triple quadrupole mass spectrometer. Phospholipids were detected using specific scans, and the quantification was based on known concentrations of the internal and external synthetic lipid standards run together with the samples.

3.7 LIPID MEDIATOR AND PATHWAY MARKER ANALYSIS

The LMs were analysed from platelet concentrates and EVs, and similarly to phospholipid profile analysis, 5 platelet concentrates were used to isolate both sample types. The LM and pathway marker sample preparation involved sample spiking with internal standards (the basis of the identification and quantification), protein removal by precipitation followed by centrifugation, and after the extraction and elution of the LMs and their sulfido-conjugates (methyl formate and methanol, respectively), the samples were concentrated and injected into a liquid chromatography-tandem mass spectrometry. The identification of the LMs was based on a two-step identification process requiring a matching retention time of the sample with the identical synthetic standard and the identification of six diagnostic ions, including at least one backbone break, from the MS/MS

spectra. The chirality of pathway markers was determined from the methyl formate fraction with chiral liquid chromatography-MS/MS.

3.8 FLOW CYTOMETRY

In Study I, flow cytometry was used to compare the Di-8-ANNEPS and CD235a positivity of the naturally produced RBC EVs and the NanoE, and to assess the particle size. Using flow cytometry, a detailed characterisation of the cellular sources of platelet concentrate-derived EVs was conducted together with the determination of CD62P exposure of the platelets in Study II, whereas in Study III, flow cytometry was used to confirm that the majority of platelet concentrate EVs were derived from platelets.

3.9 NANOPARTICLE TRACKING ANALYSIS

In Study I, NTA models LM14C, NS300, and NS500 were compared by analysing a NanoE sample using identical sample preparation and software settings for data analysis. Data capture settings were 1:10000 dilution of the sample with particle-free phosphate-buffered saline, camera level 8 or 15, and capture of 3 × 90 second videos, which had been determined as optimal for the LM14C.

In Studies I-III, the EVs were quantified and their size was determined with NTA. The samples were diluted with particle-free (as verified with NTA) phosphate-buffered saline or Hepes to a dilution of 10-50 particles/frame to assure a reliable particle quantification and size determination. If feasible, similar EV samples (e.g., day 1 samples from different platelet concentrates in Studies II and III) were prepared with the same dilution to more reliably assess real differences between concentrates rather than the artificial variation possibly generated by sample dilution. Unless stated otherwise, platelet EVs were measured with the camera level 14, and RBC EV samples or NanoE were measured with the camera level 8. For the analysis, 3 videos of 90 seconds each were captured for each sample and the sample was manually mixed in between the videos.

3.10 WESTERN BLOT

In Study III, the characterisation of the EV fraction, as well as the detection of the enzymes responsible for processing GPLs to bioactive LMs was conducted with Western blot. To acquire a sufficient amount of EVs, a separate sampling was conducted, where all sample types, platelet concentrates, platelets, and EVs, were isolated from two different platelet

concentrates on day 1 and day 8. An equal quantity of samples, 25 µg of protein, was loaded together with protein size standards to wells of commercial gradient gels. After running the sample front until the end of gel, the proteins were blotted to nitrocellulose membranes using a semi-dry blotting machine. After the confirmation of successful blotting, the membranes were blocked with milk proteins.

EV samples were examined for the presence of apolipoproteins, platelet marker CD41, and EV markers CD9 and CD63, and the presence of different members of PLA, LOX, COX and CYP family enzymes in all sample types was assessed with an overnight primary antibody incubation at +4 °C. After washes, an hour of secondary antibody incubation at a room temperature, and addition of the enhanced chemiluminescence reagents, the antibody-specific chemiluminescence was captured.

3.11 STATISTICAL ANALYSIS

In Study I, the differences in the Di-8-ANNEPS lipid dye labelling and the CD235a-labelling of RBC EVs and NanoE was determined using paired two-tailed t-test.

In Study II, the significance of the alterations in the assessed platelet activation markers within one PAS were determined using Kruskal-Wallis test together with Dunn's multiple comparison test. The differences between PAS on day 5 samples were assessed using single Mann-Whitney test together with Bonferroni correction for each platelet activation marker.

In Study III, the significance of EV accumulation to platelet concentrates and time-dependent alteration of the EV size profile were determined using Friedman's test with Dunn's multiple comparison test. The significance of the variation in the relative quantities of phospholipids and pathway markers or LM quantities was assessed using Friedman's test with Dunn's multiple comparison test. The differences in phospholipid profiles of platelet concentrate, platelets, and EVs were analysed using principal component analysis (PCA) in Study III. In all experimental settings, p-values < 0.05 were considered statistically significant. Statistical analysis was conducted using GraphPad Prism (GraphPad Software, San Diego, CA, USA), PCA visualisation was done using Metaboanalyst [349].

4 RESULTS

4.1 NANOERYTHROSOMES AS A REFERENCE MATERIAL

As the synthetic reference materials are comparable to EVs only in certain aspects, e.g., nominal diameter, and many of their properties, e.g., the refractive index, which is crucial in flow cytometry, do not resemble EVs, a biological reference material was developed to improve the comparability and traceability of EV studies. The development of the biological reference material was initialised with a thorough literature search to map candidates categorised as naturally occurring particles (EVs, lipoproteins, viral particles, coccoid-shaped organisms (Table 3 in Study I)) and as particles requiring some production or manufacturing steps (disrupted cells or lipid constructs (Table 4 in Study I)).

Based on the query conducted during the Study I in 2014, the most commonly used technique in EV studies was flow cytometry, and as polystyrene and silica beads are commonly used in flow cytometry, over 50% of the responders indicated the use of synthetic reference materials in their EV studies. On the basis of the questions about biological reference material, the most appreciated properties of biological reference material were stability and biochemical composition reminiscent of natural EVs, and 80% of the replies indicated the need for phospholipid membrane and proteins. If safety could be guaranteed, 60% of the respondents indicated readiness to use plant viruses or marine bacteria as the reference material for EVs (Fig. 2 in Study I).

To fulfil the desired requirements, the reference material was prepared from disrupted cells. The tested methods to produce nanoparticles from cells varied greatly in their efficacy to break down the cells, as evident by EM. Freeze-thaw cycles did not disrupt the RBCs but only made the cells leaky, as the visibly red haemoglobin content could be washed away, resulting in colourless pellet contrary to red pellet after the sonication of RBCs. The N_2 bomb disrupted the cells completely to membrane fragments of various sizes, but these fragments could not be resealed to form spherical particles (Fig. 3 in Study I). Ultrasonication of isolated RBCs resulted in nanoparticles morphologically similar to RBC EVs (Fig. 9A and B). Further comparison of NanoEs and RBC EVs proved that the particles were similar in terms of size and refractive index, as approximately 80% of the particles were 100-300 nm in diameter and approximately 70% of the particles had a refractive index of 1.35-1.37 (Fig. 9C and D). However, molecular differences were also apparent, as differences in CD235a expression, Di-8-ANNEPS positivity, and general protein composition could be observed between NanoE and RBC EVs (Fig. 9E and F).

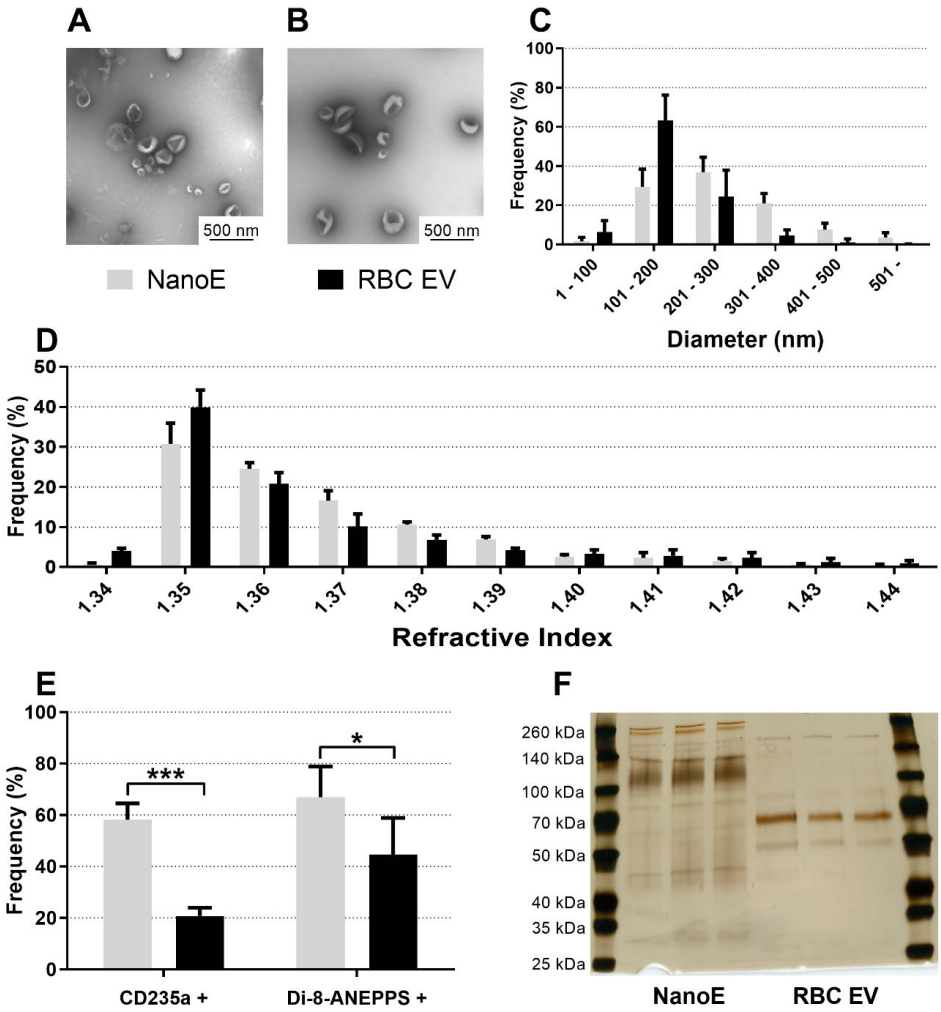

Figure 9: Comparison of nanoerythrosomes (NanoE) and red blood cell-derived extracellular vesicles (RBC EV) showing transmission electron microscopy micrographs from [A] NanoE and [B] RBC EV, [C] size distribution, [D] refractive index distribution, [E] CD235a and Di-8-ANNEPS positivity, and [F] silver-stained SDS-PAGE gel of NanoE and RBC EV. Columns represent mean and bars standard deviation, n = 3 [D], 6 [E], or 18-20 [C]. *$p \leq 0.05$, ***$p \leq 0.001$ using paired t-test. Figure compiled from the Figures 4-6 in Study I.

Currently, EVs are assessed with various methods and alarmingly, even with a single technique, NTA, using three different models with similar hardware and identical sample preparation, measurement settings, and data analysis, the sample quantification analysis might result in completely different results, as shown in Figure 10.

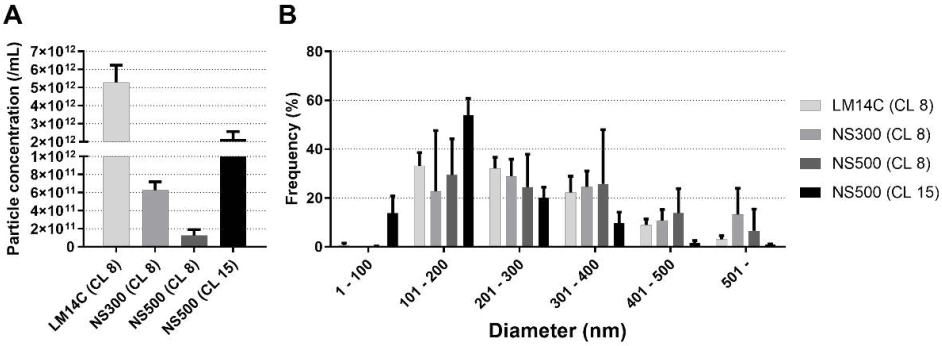

Figure 10: [A] Particle concentration and [B] size distribution of identical nanoerythrosome samples measured with Nanoparticle Tracking Analyser models LM14C, NS300, and NS500 using either the camera level (CL) 8 or 15. Columns represent mean and bars standard deviation, n = 3. Figure modified from Figure 7 in Study I.

The result indicates that despite the seemingly identical measurement settings and samples, the determined particle concentration and size distribution differed solely due to the used instrument: sample analysis with LM14C resulted in the highest particle concentration, as approximately a 10-fold higher particle concentration was observed compared to sample analysis with NS300 and NS500. However, the detected size distribution of the particles was similar with all the measurements. When the NanoE sample was analysed with NS500 using more optimal settings for the given sample, the camera level 15 in the case of this instrument, a particle concentration closer to the LM14C measurements was obtained (unpublished result). Still, the determined size distribution measured with the optimal settings for NS500 differed from the results for the other measurements, as the relative amount of particles with the diameter less than 100 nm was notably increased and consecutively larger particles were detected less.

4.2 EXTRACELLULAR VESICLES AND PLATELET ACTIVATION

Platelets secrete EVs as part of their homeostasis, and upon activation, the EV production is increased. As part of the study, the EVs of ageing platelet concentrates were quantified and characterised to determine how the storage of platelets influences the EV population in the platelet concentrates. Besides the effect of the ageing of platelets, the effect of storage conditions on the EV secretion of platelets was assessed by comparing the EV populations isolated from platelet concentrates with different PAS.

A significant increase in the platelet activation status was observed with all the assessed platelet activation markers (CD62P exposure of platelets, sGPV, and EV count) by day 5 in platelet concentrates with PAS-B (Fig. 11A-D). In PAS-E concentrates, a significant increase in the platelet activation status was detected starting from day 5 with the sGPV and EV count. However, using CD62P exposure, a significant increase in the platelet activation was detected only in day 8 samples. The analysis of day 5 platelet concentrates indicated that platelets stored in PAS-E containing potassium, magnesium, and phosphate are activated to a lesser degree, which was also indicated by the lower CD62P exposure of PAS-E platelets. Temporal change in the EV numbers of the platelet concentrate was shown to serve as an accurate biomarker for platelet activation since the fresh and expired platelet concentrates and even concentrates with different PAS could be differentiated based on the EV concentration. Most importantly, a change in EV concentration correlated with changes in the established platelet activation markers, underlining the feasibility of applying the EV concentration as a marker of platelet activation (Fig. 2 in Study II).

In addition to the EV count, the platelet storage conditions also influenced the size distribution of the secreted EVs: EVs with a larger diameter were secreted from platelets stored in PAS without magnesium, potassium, and phosphate compared to the platelets stored in PAS with aforementioned components (Fig. 11E), as the modal size class of EVs was changed from < 100 nm EVs to 101-200 nm EVs by day 8.

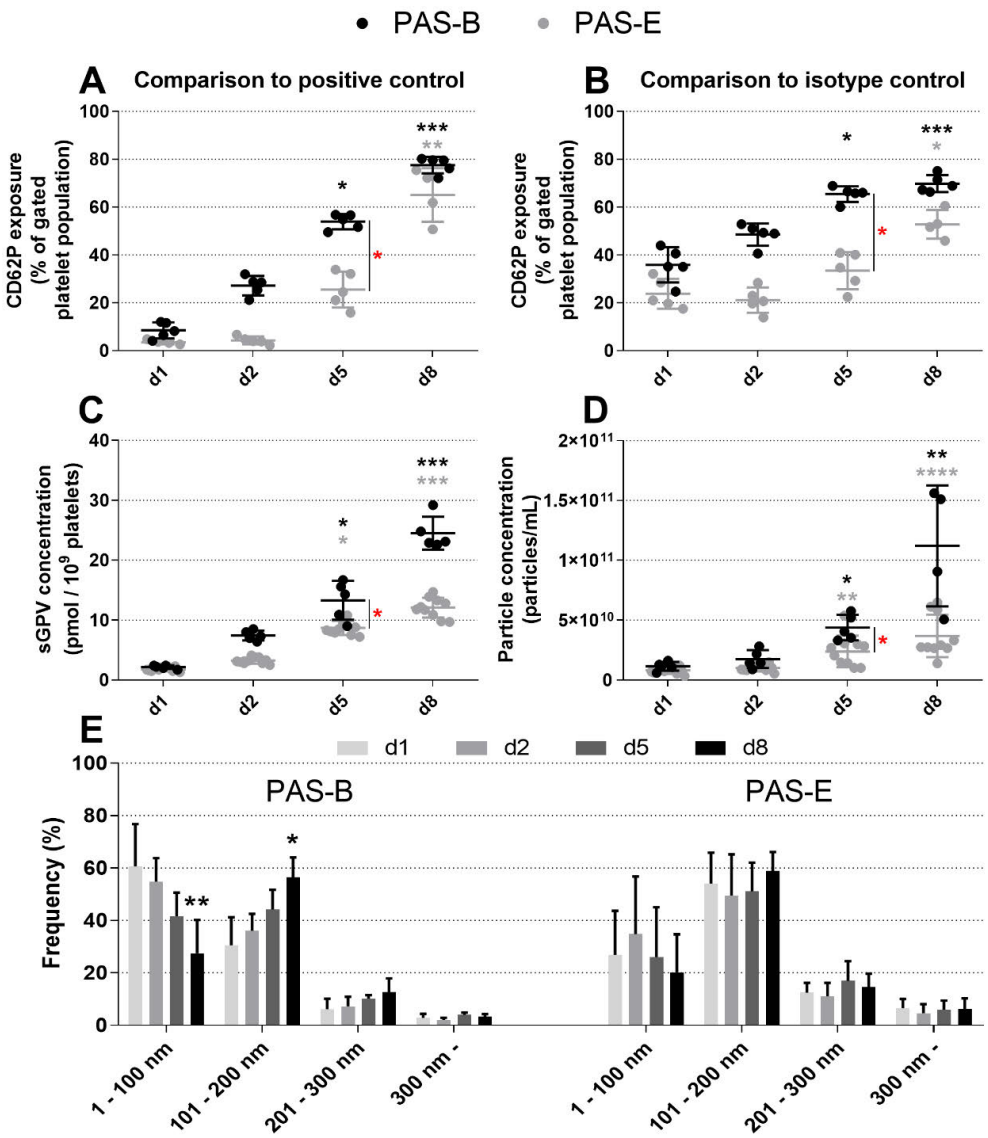

Figure 11: Quality control markers used for the evaluation of platelet activation during storage of platelet concentrates with platelet additive solution (PAS) B and PAS-E. Time-dependent changes in the CD62P exposure of platelets, when compared to [A] thrombin-activated positive control or [B] isotype control, [C] soluble glycoprotein V (sGPV) production of platelets, [D] concentration and [E] size distribution of particles in the extracellular vesicle samples isolated from platelet concentrates. Statistical difference within a given PAS is indicated with black and grey stars for PAS-B and PAS-E platelet concentrates, respectively, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ compared to day (d)1 using Kruskal-Wallis test with Dunn's multiple comparison. Statistical difference between PAS-B and PAS-E platelet concentrates on d5 is indicated with a red star, *$p \leq 0.05$ using Mann-Whitney test with Bonferroni correction. Bars represent mean with standard deviation in A-D, columns present mean and bars standard deviation in E. Data were acquired in 3 independent experimental settings, n = 4-5 [PAS-B in all figures, PAS-E in A and B] or 10 [PAS-E in C, D, and E]. Figure reproduced from Study II with permission from Karger AG.

4.3 LIPID-MEDIATED SIGNALLING OF EXTRACELLULAR VESICLES

One crucial milestone of the EV field was the discovery of EVs' capability to transport RNA later transcribable to functional proteins in target cells. Although this significant finding revealed a new dimension of intercellular communication, it is only one mode of communication in the multifaceted interaction skills of EVs. In Study III, EVs' potential contribution to the platelet function via lipid-mediated signalling was assessed in the specific, isolated environment of platelet concentrates.

The assessment of the compositional changes in EVs from platelet concentrates demonstrated a time-dependent alteration on phospholipid class level (Fig. 12, unpublished data), a significant time-dependent decrease in the relative amount of PC is reflected by the increase in the relative amount of PS, PE, PE plasmalogens, and lysoPC.

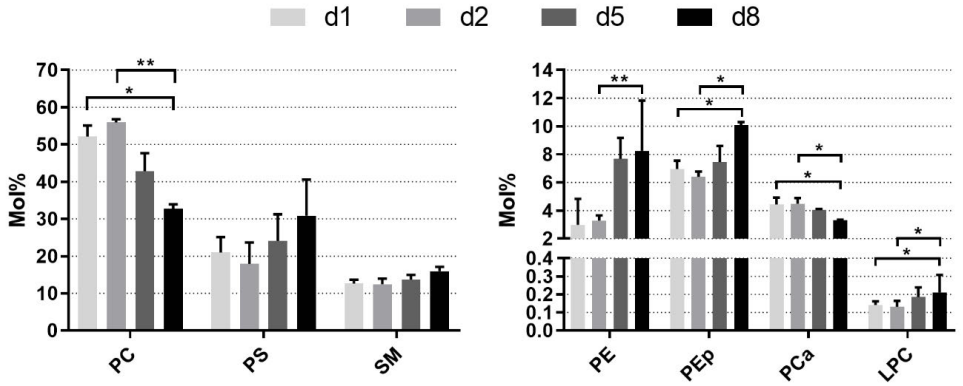

Figure 12: Time-dependent phospholipid class level alterations of the extracellular vesicles from 5 platelet concentrates on days (d)1, d2, d5, and d8. Columns represent median and bars interquartile range. *$p \leq 0.05$, **$p \leq 0.01$ using Friedman's test with Dunn's multiple comparisons test. PC = phosphatidylcholine, PS = phosphatidylserine, SM = sphingomyeline, PE = phosphatidylethanolamine, PEp = phosphatidylethanolamine plasmalogen, PCa = phosphatidylcholine alkyl-acyl, LPC = lysophosphatidylcholine.

When the lipidome of EVs was examined in more detail at the phospholipid species level, it could be observed that initially, platelets secreted EVs with relatively short and highly unsaturated PE, PE plasmalogen, and PS, but upon storage, the type of EVs secreted by the platelets changed. In practice, EVs with an increased relative amount of AA-containing species became dominant, which was particularly evident in the PE and PS classes (Fig. 13). Although alterations in the relative phospholipid profile were observed at both class and species level of EVs, virtually very little time-dependent alteration was observed in the FA profile of the platelet concentrates (Supplementary Fig. 2 in Study III).

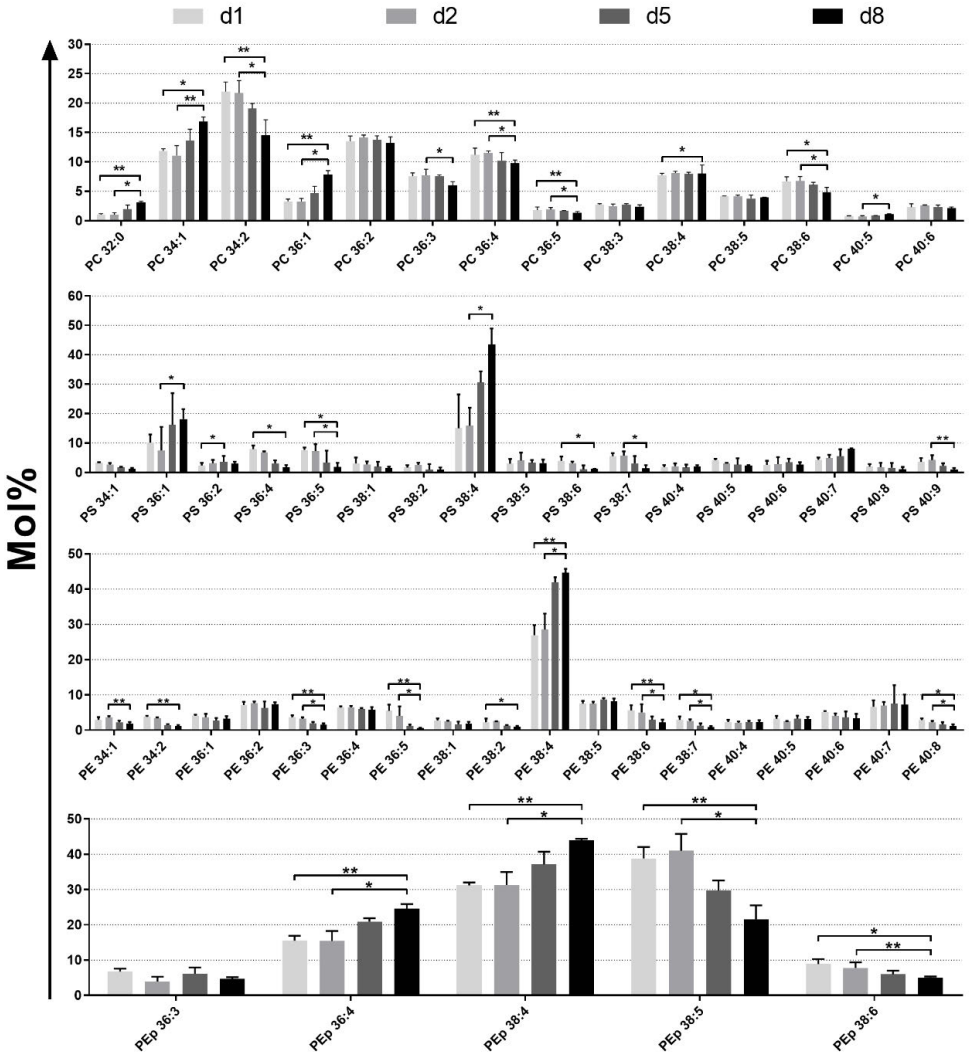

Figure 13: Relative abundance of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylethanolamine plasmalogen (PEp) species of extracellular vesicles from 5 platelet concentrates on days (d)1, d2, d5, and d8. Columns represent median and bars interquartile range. *$p \leq 0.05$ **$p \leq 0.01$, using Friedman's test with Dunn's multiple comparisons test. Figure compiled from the Figures 2-4 and Supplementary Figure 3 from Study III.

When the phospholipid profile alterations were visualised using PCA, the time-dependent compositional changes of EVs were also apparent, as the day 1 and 2 samples, as well as day 5 and 8 samples, formed their separate subpopulations. Such variations were not observed in the tightly clustered populations of platelet concentrate and platelet samples. Moreover, the phospholipid profiles of EVs were different from the phospholipid profiles of whole platelet concentrate and isolated platelets, as the confidence intervals of different sample populations did not overlap (Fig. 14).

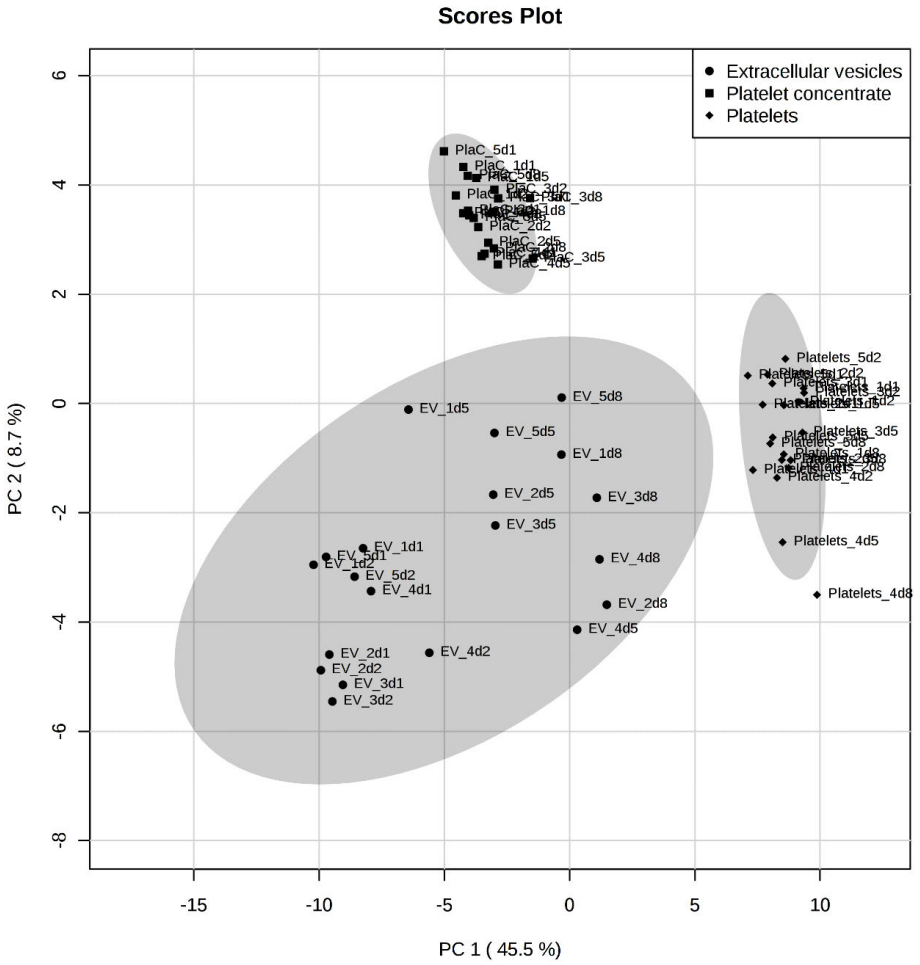

Figure 14: Visualisation of principal component analysis of phospholipid composition in the platelet concentrates, platelets, and extracellular vesicles. All quantified phospholipid species were used as loadings. Samples were assigned codes consisting of the sample type (PlaC/Platelets/EV), platelet concentrate number (1-5), and the sampling day (d1, d2, d5, or d8); shaded areas represent 95% confidence intervals. Figure reproduced from Study III with permission from Elsevier.

Interestingly, EVs were further shown to contain the required enzymatic machinery to produce bioactive LMs (Fig. 15A): Especially in the day 8 samples with the highest EV content (Fig. 1 in Study III), cytosolic and secretory PLA_2, but also 12-LOX, 15-LOX2, COX1, (CYP)1A1, and CYP5A1 enzymes were detected by Western blot. The increased CD41 in the EV samples is in line with the results observed in Study II showing temporal EV release in the platelet concentrate. A similar increase was not detected in EV markers CD9 and CD63, and the lipoprotein contamination in EV samples also remained stable. The functionality of enzymes in platelet concentrates was verified indirectly through the examination of the chirality of pathway markers showing the prevalence of either *S* or *R* configuration of the pathway markers (Fig. 15B).

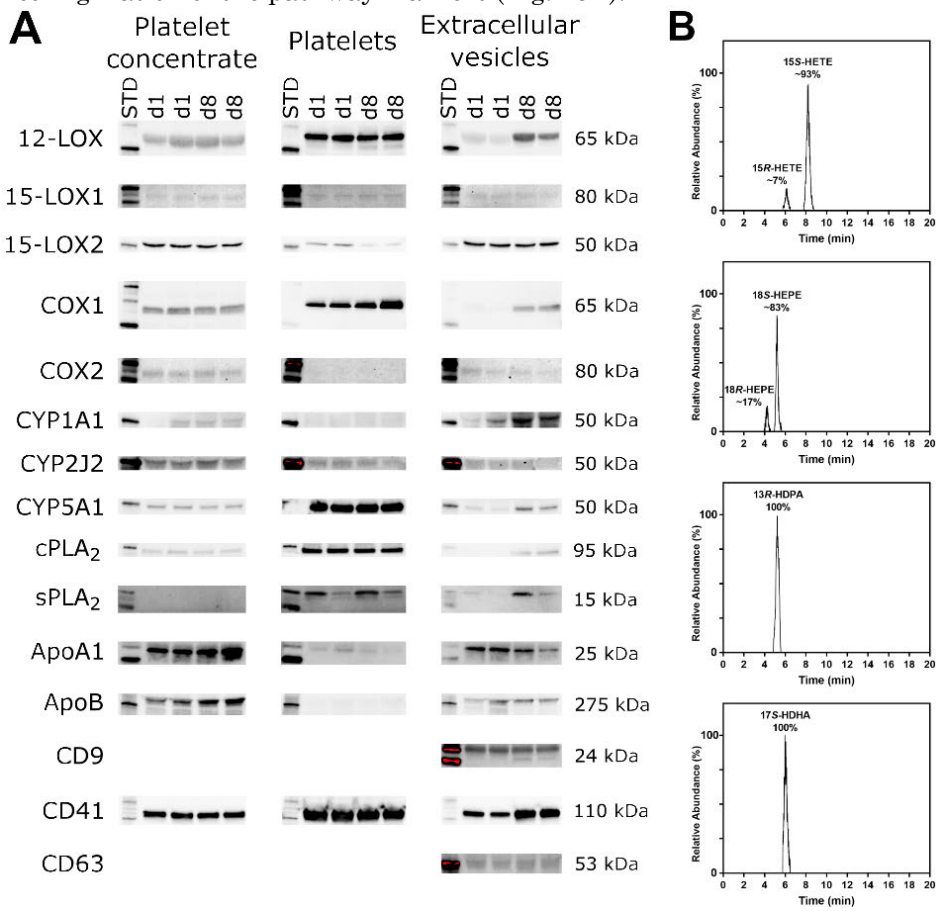

Figure 15: [A] Enzyme detection from the platelet concentrates, platelets and extracellular vesicles by Western blot and [B] chirality analysis of monohydroxy pathway markers. 12-lipoxygenase (LOX), 15-LOX1, 15-LOX2, cyclooxygenase (COX)1, COX2, cytochrome p450 (CYP)1A1, CYP2J2, CYP5A1, cytosolic phospholipase 2 ($cPLA_2$), secretory phospholipase 2 ($sPLA_2$), CD41, apolipoprotein (Apo)A1, and ApoB were detected from the platelet concentrates, platelets and extracellular vesicles. Samples were isolated from two different platelet concentrates on day (d)1 and d8. The size of detected proteins is indicated on the right side of the figure, STD = molecular weight standard. Figure reproduced from Study III with permission from Elsevier.

Besides FAs and enzymes for processing FAs to bioactive LMs, EVs also contained proinflammatory LMs (TxB_2 and PGD_2) and pathway markers (12-hydroxyeicosatetraenoic acid (HETE)), dual-function LMs (PGE_2) as well as SPMs (17*R*-RvD1) and proresolution pathway markers (14-hydroxydocosapentaenoic acid (HDPA)) in quantifiable amounts. The EV fraction also contained LMs unique to the EV fraction (SPMs RvE3, RvD3, and MaR1). Some LMs and pathway markers (e.g., TxB_2 and 4-hydroxydocosahexaenoic (HDHA)) were steadily expressed in EVs, whereas the amount of some (e.g., PGE_2 and 12-HETE) clearly demonstrated a time-dependent accumulation in the EV fraction (Fig. 16; Supplementary Table 2 in Study III).

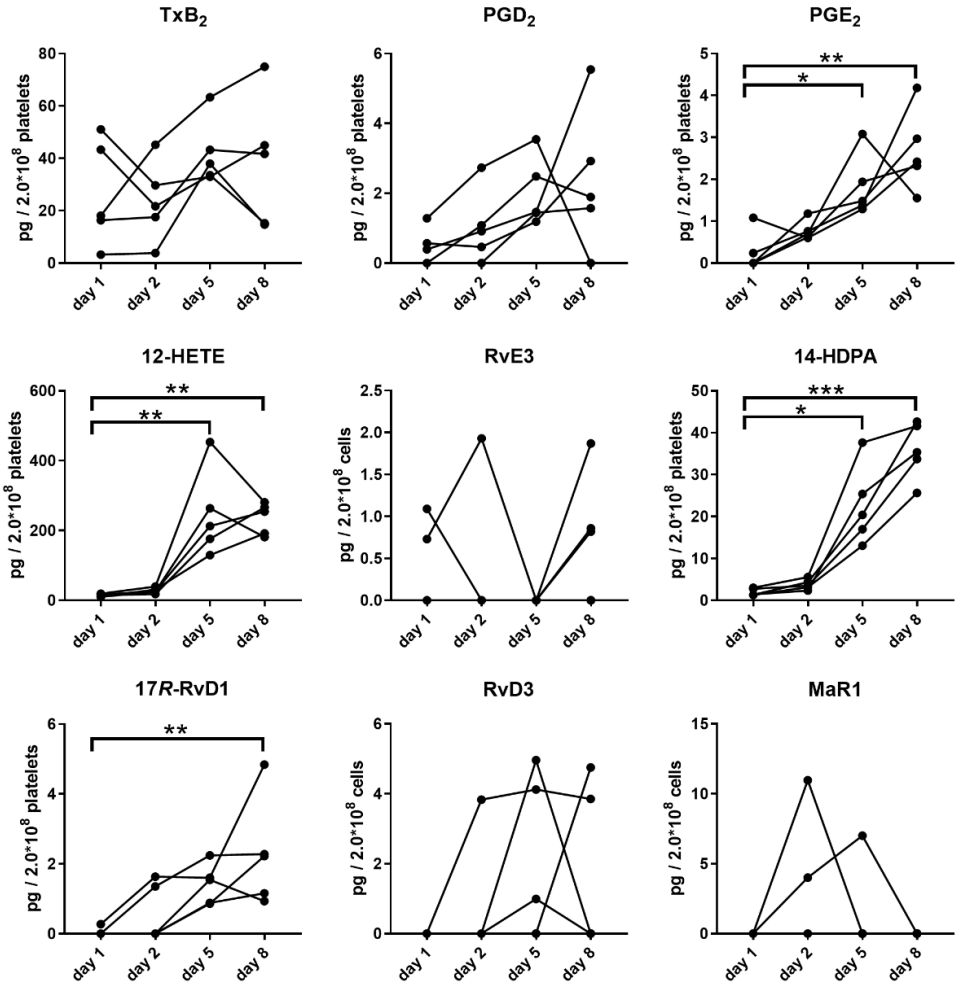

Figure 16: Accumulation of lipid mediators and monohydroxy pathway markers in the extracellular vesicles of 5 platelet concentrates during storage. Results are expressed as pg / 2.0 × 10^8 platelets. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ vs. day 1 using Friedman's test with Dunn's multiple comparisons test. HDPA, hydroxydocosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; Mar, maresin; PG, prostaglandin; Rv, resolving; Tx, thromboxane. Figure compiled from Figure 8 and Supplementary Table 2 from Study III.

5 DISCUSSION

5.1 ON THE ROAD TO TRACEABLE EXTRACELLULAR VESICLE ASSESSMENT

In the vigorously developing EV field, optimal golden standards regarding isolation, quantification or characterisation remain to be developed and disseminated, and in the meanwhile, EVs are assessed with various techniques. The results of this study underscore the instrument dependency of even simple EV quantification and particle sizing, as already with one type of a device the sample analysis may result in e.g., a 10-fold variation in particle concentration. An important question then arises: how to compare any EV results analysed by different techniques and laboratories? To construct an accurate understanding of how EVs are involved in intercellular communication on a molecular level, results from multiple sources achieved with various techniques need to be compared. Only a thorough comprehension of the mechanisms behind EV functions will enable further exploitation of EVs in various theranostic applications, therefore repeatable and comparable experiments are of paramount importance.

If the pitfalls in sample isolation are excluded, then a thorough answer on how to address measurement-based discrepancy of EV results can be divided into three parts. Firstly and maybe most importantly, method development should aim to produce techniques addressing the entire EV population, and not only part of it, by taking into account the physicochemical properties that are currently understood the best e.g., the low refractive index of EVs compared to synthetic particles and the small diameter of EVs. Although this sounds simple, the task is not that easy to carry out, therefore for the time being EV quantification and characterisation will continue to be conducted with combinations of different kinds of techniques that are currently available.

Secondly, one cannot stress enough the importance of a thorough comprehension of the used technique and especially its shortcomings: with NTA, the measurement settings reported in previous publications should not be used as a point of reference in the measurements, as this study points out that even the outcome of identical measurements with similar instruments differ. Strikingly the variation in the detected particle concentration was 10-fold, but the determined sample size distribution was similar with all instruments. As the same sample dilution was used throughout the measurements, the particle count during the data acquisition was approximately 10 times lower in measurements conducted with NS300 and NS500 compared to LM14C. Such a low particle count is inadequate for reliable sample analysis as it is below the linear range

of the technique [254], influencing the determination of both particle concentration and size distribution [250]. Indeed, when the sample analysis was conducted using NS500 with optimal settings for it, the particle concentration was more similar to the particle count detected with LM14C using its optimal conditions. However, the particle size distribution detected with NS500 using optimal settings differed from the size distribution obtained with the camera level 8 setting (regardless of the instrument model) as notably more < 100 nm particles were detected in relation to other particles. The result demonstrates that the same sample is perceived differently even by the same technique and seemingly identical hardware, as it seems that with optimal measurement settings, NS500 detected small particles with better efficacy than LM14C. Since the data analysis was conducted with the same software version, these results pinpoint one crucial source of variation in the EV studies performed with NTA (Nanosight).

To minimise measurement errors, measurements should be conducted with individually tailored conditions ideal for the particular sample type to assure that the sample analysis is performed in the best possible manner given the known limitations of the technique. In practice, with regard to NTA (Nanosight), this may even require separate measurements for the optimal determination of particle concentration and size distribution, as high particle count during data capture results in more reliable determination of size distribution, while possibly leading to erroneous determination of particle concentration due to particle masking [254], which can be detected by measuring a dilution series of the sample [350].

The third part of the answer is to improve the comparability of the results by creating a common denominator for the measurement results, a reference material. International Vocabulary of Metrology defines reference material as "material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties" [351]. For instance, in another popular method for EV characterisation which may be applied to quantification, flow cytometry, synthetic references, typically polystyrene or silica beads, have commonly been used to estimate the size of a target population, either cells or EVs [352]. However, the use of synthetic reference material may lead to underestimation of the EV size and even assessment of wrong particle population (because cellular debris could be interpreted as EVs), as the refractive indices of polystyrene (1.59) and silica (1.45) notably differ from the refractive index of EVs (~1.39) [254,353], and EVs would have to be notably larger than synthetic beads to scatter a similar amount of light and to be detected with gating of the synthetic reference materials [352]. Due to this and the relatively high detection limit of older generation flow cytometers [352], together with swarm detection of EVs, erroneous

interpretations of EVs have been made. For example, the impression that platelet-derived EVs constitute the majority of plasma EVs [243] is predominantly due to the gating of EVs that are much larger than what is currently known to be the size of the major pool of EVs, < 300 nm.

From the perspective of physicochemical properties, ideal reference material for EV studies would be comparable to EVs and characterised in detail with a metrologically traceable method. Metrological traceability is defined as "the result should be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty" [351]. Such reference material would enable a reliable point of comparison for multiple different techniques (and with different instrument models), ensuring complete transparency and comparability of experiments. A biological reference material similar to EVs would benefit the EV field in many ways: importantly, as EV research is a rapidly developing and highly method-dependent field, in the area of technology development, the optimisation of instrument settings would benefit from a standard reference material with EV-like properties [354]. Another way to universally apply reference material to EV quantification is to use it as a calibrator of a method [254,355]: After carefully quantifying the reference material by a metrologically traceable method, the accuracy of different quantification techniques, instruments, and quantification measurement rounds can be determined by comparing whether the measured concentration of reference differs from the concentration of the well-characterised reference material concentration achieved with a metrologically sound method, enabling a calibration factor to be calculated and applied to the obtained results.

Ultimately, EV measurements need to be metrologically traceable if the use of EVs in e.g., theranostics is desired. To properly represent EVs, a close similarity to EVs is required from the reference material in terms of physicochemical properties: Most importantly, the reference material should be safe to use. An ideal reference material for EV studies should consist of monodisperse particles with a similar size to the major population of naturally occurring EVs, approximately 300 nm, and EV-like refractive index of 1.38. Regarding structural requirements, the reference material should contain surface proteins and genomic material. For the reference material to be widely applied, it should be either affordable to acquire or otherwise accessible by e.g. mass-production of stable lots of particles (Fig. 17).

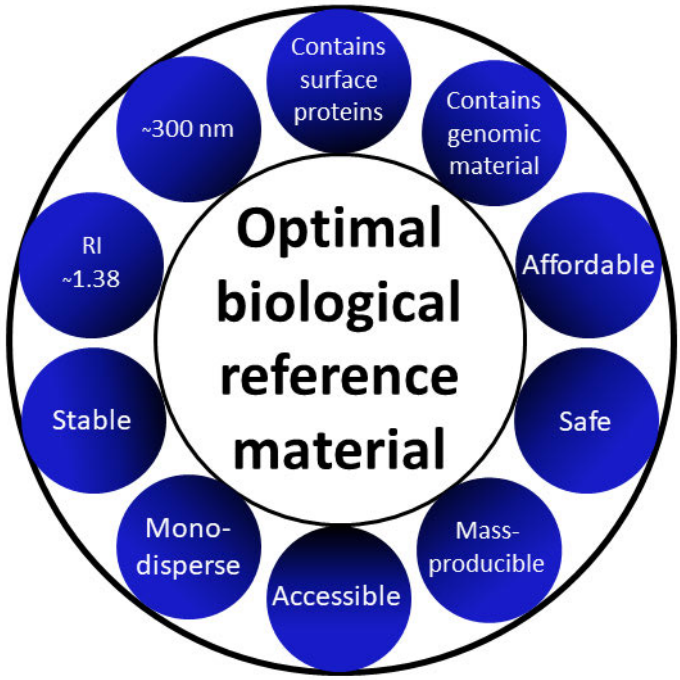

Figure 17: Desired properties of reference material applicable for multiple techniques in extracellular vesicle studies. Reproduced from Study I with permission from Elsevier. RI = refractive index.

However, as long as the differences between the standard and a sample are known, and the limitations of the measuring techniques are acknowledged, a simpler standard can still be utilised for a limited number of applications. For example, if an EV reference is developed to address only one EV quantification or characterisation technique, e.g., flow cytometry, fewer physicochemical properties of particles need to be taken into account, and a more optimal reference for a specific technique could be used or developed *de novo*. Examples of such reference materials for flow cytometry include monodisperse hollow silica particles or engineered retroviruses [255,356], which do not resemble EVs in most physicochemical properties, but from the perspective of light scattering and monodispersity they fulfil the criteria of a good reference material tailored for flow cytometry.

Keeping these caveats and aspirations in mind, an attempt was made in this study to genuinely increase the comparability of EV results by investigating the properties required of a biological reference material and by generating a candidate that would be suitable for multiple EV quantification and characterisation techniques. After careful consideration (via a questionnaire and a literature search), a decision was made to generate the candidate from RBCs because of the readily available material and the robustness of structurally simple and well-characterised RBCs. One crucial property of RBCs as a source for reference material is

their exclusive expression of CD235a [357], providing trackability for the reference material [355], as CD235a expression enables reference material identification from a pool of natural EVs (excluding RBC EVs), which may enable a more complex use of the reference. EV sample spiking with reference material expressing CD235a could be applied to the determination of e.g., the effectiveness of an EV isolation procedure [185,355] or to the relative quantification of EVs using flow cytometer by mixing a known amount of reference material to EV sample and determining the ratio between CD235a-positive particles and the original EV sample particles. Especially the biochemical similarity and stability were also considered to be desired properties for a reference material in the questionnaire sent to 50 laboratories working with EVs.

Then, the mass production of reference material was assessed and as a result, NanoEs were produced from RBCs via sonication and the method was optimised. A major advantage of NanoE as a reference material, compared to isolated subpopulations of EVs, is the production cost: in a tube of blood, approximately half of the volume consists of RBCs, and the NanoE production utilises the entire lipid membrane of RBCs. In practice, this means that from 10 mL of whole blood, one can prepare a 4-5 mL pellet of NanoEs in a matter of hours with only basic laboratory equipment. Isolating the same amount of plasma EVs would require litres of blood and countless hours of centrifugation, or alternatively, if cell line-derived EVs were sought for, multiple high-yield 3D bioreactors [358,359] with high initial costs, and weeks of EV harvesting, not forgetting the additional task of EV isolation. As another evident advantage of the simple production method and readily available materials, NanoEs could be reproducibly generated in different laboratories independently in contrast to the highly variable results of EV isolation.

Besides NanoEs' potential as biological reference material, their production concept offers an interesting opportunity to study in detail the molecular enrichment in EVs through comparison of naturally produced particles and manufactured particles from the same source. As NanoE production is a relatively straightforward mechanical procedure involving the breakdown of RBCs to smaller particles, the molecular composition of NanoEs should resemble the natural distribution of lipids, proteins, and carbohydrates of the RBC membrane, but on a nanoscale. The EV composition differs from the natural composition of cells [360,361], as also shown by the results of this thesis (Fig. 14), and furthermore, the composition is, at least in the case of platelets, activation-dependent [169–172]. Analogously, the NanoEs, a miniature representation of plasma membrane, were not identical to naturally produced RBC-derived EVs. As the protein and lipid composition of NanoEs was different from that of RBC EVs, the differences are most likely applicable to the molecular composition in general. These results also indirectly indicate the specific cargo loading to EVs, and the compositional differences are presumably

also reflected in functional differences. Therefore, a detailed molecular characterisation of RBC EVs and NanoEs, together with the comparison of different functional aspects, such as cellular uptake, may facilitate the identification of the moieties responsible for different functions. With a similar method, platelet-derived nanoparticles could also be produced [362], and therefore it can be deduced that cell disruption using ultrasonication can be applied to various cell types, enabling a detailed pair-wise comparison of natural EVs and nanoparticles produced from corresponding cells. As a curiosity regarding platelet-derived EVs, calcium ionophore induces EVs shown to be unlike natural platelet-derived EVs [169], therefore the comparison of nanoparticles produced by platelet sonication and calcium ionophore-induced platelet-derived EVs would be highly interesting. With this approach, the unique cell type-specific EV functionality could be examined accurately, allowing the identification and comprehension of such moieties better, which would ultimately facilitate the development of different EV-based applications.

As a clear limitation of Study I, only a superficial analysis of the lipidome and proteome was conducted, and the RNA content of NanoE was not assessed at all, therefore the further detailed characterisation of NanoE is necessary. As indicated in the questionnaire, the presence of genomic material was considered to be a desired property for reference material. As RBCs contain RNA [363], it could be presumed that NanoE produced with the method described in Study I may contain some RNA, which would enable further applications of the reference material, e.g., the determination of whether RNA is intact after general sample treatment, such as sample isolation or extended storage of samples. The minor difference in the refractive index of NanoE and RBC EVs can most likely be resolved by washing the contents, e.g., haemoglobin, of the visibly red NanoE (when produced with the method reported in Study I): After initial RBC isolation the cells should be subjected to three freeze-thaw cycles. As the freeze-thaw treatment does not break down the RBCs (Fig. 3A in Study I), but render the RBCs leaky, the freeze-thaw treatment of RBCs enables the RBC content washing, after which ultrasonication could be used to break down the washed RBCs to NanoE. Since the contents of particles also influence the total refractive index of the particle [255], arguably preparation of NanoE from the emptied RBCs in aqueous solution with a lower refractive index would result in NanoE having a lower overall refractive index and therefore an even closer resemblance to RBC-derived EVs. Another significant way to improve NanoE as a reference material would be to further modify the NanoE manufacturing protocol for instance by applying different ultracentrifugation protocols to especially isolate smaller particles or by adding phospholipids before sonication to influence the diameter of particles formed as a result of ultrasonication [364]. A proper reference material should contain monodisperse populations of particles, and with NanoEs this is could be achieved by applying simple

filtration or latest isolation techniques, such as asymmetric-flow field-flow fractionation [227], to crude NanoE preparation. Given the mass production possibility of NanoEs, even commercial amounts of preparations with particle populations of specific sizes could be prepared without problems. Ideally, EV reference material would consist of particles covering the size range of the majority of the EV population, and for example, with NanoE, the preparation of particle subpopulations with the diameter of 100, 200, and 300 nm would be possible.

To summarise, RBC-derived NanoEs represent an accessible, affordable, and mass-producible reference material that is safe to use. The characterisation of NanoEs proved that the produced particles were similar to naturally produced RBC EVs in many physicochemical aspects and stable over extended storage, therefore it can be concluded that in many ways RBC-derived NanoEs fulfil the criteria for a good reference material for EV studies (Fig. 17), or that they can be further refined to meet these criteria. After minor improvements and detailed characterisation to formally assess the level of similarity to naturally occurring EVs, NanoEs could enable metrologically traceable EV measurements, on condition that the reference material is accessible to the EV field and incorporated routinely into EV measurements. The NanoE represents an important advance in the standardisation of the EV field and, at the time of starting this thesis project, similar studies had not been published. Recently, however, another alternative for a biological reference material, recombinant EVs, has also been published [355].

Finally, even if a perfect reference material enabling metrologically sound assessment is developed for EV studies, rather than remove the shortcomings between different techniques or the differences in measuring sensitivity, it would make the results comparable and point out the variance between methods. Therefore, the EV field would benefit most from a methodology with more uniform detection of EVs. However, having a well-characterised EV reference material such as NanoE may also facilitate method development and optimisation.

5.2 THE EXTRACELLULAR VESICLES OF PLATELET CONCENTRATES: THE GOOD, THE BAD, OR THE UGLY?

Blood transfusions are aimed at restoring the platelet and RBC counts to the physiological level, which is an effective countermeasure against anaemia and bleeding. However, simultaneously EVs are also transfused to patients. Previously, the EVs of blood concentrates were mostly overlooked, but more recently the possible roles of EVs in transfusions have also become better recognised (reviewed in [365,366]): the EVs of platelet concentrate have been hypothesised to contribute to haemostasis and influence the functionality of the other cells in the circulation, which adds a level of complexity to consider, particularly in the transfusions of older blood products containing more EVs (Studies II and III) with the potential to rapidly influence the blood cells e.g. via lipid-mediated signalling (Study III).

It is fascinating to think that even though the platelets and leukocytes constitute less than 6% of the cells in circulation [10], approximately 30% of the plasma EVs are derived from these cells [104]. By now, it has been well established that EVs are not senseless bubbles randomly floating around, but rather messengers with a purpose, and the logical next step also in the transfusion field is to try to understand how to translate the important messages conveyed by EVs with current quantification and characterisation methods.

In platelet concentrates, the EV count was found to be a sensitive measurement for determining platelet activation, with sensitivity comparable to that of the currently existing platelet activation markers widely used in transfusion clinics. Also, using EV concentration, the assessed platelet concentrates with different PAS could be differentiated based on the extent of platelet activation on day 5, the last day when the concentrate is still transfusable to patients, underlining the sensitivity of platelets and their reactivity to the exposure to different conditions. The difference in platelet activation in PAS-B and PAS-E concentrates on day 5 was equally well demonstrated with all examined readouts (EV count, sGPV production, and CD62P exposure of platelets). Taken together, these results on platelet-derived EVs are in line with previous experiments showing the effect of platelet-activating conditions on secreted EVs [169–172], the time-dependent increase in EV content of platelet concentrates [367–369], the time-dependent activation of platelets in platelet concentrates [30,38], and the relationship between the composition of PAS and platelet activation [370–372]. As EVs are a fundamental part of platelet functionality, EVs genuinely are a valuable indication of the

platelet status and provide information that complements the currently existing platelet activation markers.[1]

A good, upscalable quality control marker for blood products would be such that it requires minimal sample preparation and does not need immediate analysis after sampling, and the quantitative parameter should be determinable with an automated and standardised system requiring as little hands-on time as possible [38]. CD62P fulfils these criteria in many ways, as only a small (unprocessed) sample from platelet concentrate is needed, a positive control can be prepared, and the sample labelling and processing are simple, but as a downside samples have to be processed fast to prevent unspecific artificial platelet activation [30]. Another possible source for variation is the cleavage of CD62P from the platelet surfaces to soluble form [373]. The use of sGPV as a platelet activation marker requires moderate sample preparation to get a platelet-free supernatant, but the samples are stable in extended storage and the platelet activation state can be determined accurately using a positive control or the known amount (12 000 copies/platelet) of the receptor on a platelet and total amount of platelets in the sample [38]. However, a clear pitfall of the sGPV determination is the actual quantification performed with an enzyme-linked immunosorbent assay, as previously the commercial kit was available only from one manufacturer. Platelet activation can also be determined from other soluble components (reviewed in [374]), and one option would be the quantification of soluble CD62P [373]. Similarly to sGPV, approximately 13 000 copies of CD62P are expressed on the platelet surface [375], but the major drawback of soluble factors is that the sample preparation involves the preparation of platelet-free and also EV-free supernatant, as platelet-derived EVs express CD62P [373].

EVs, on the other hand, require extensive sample preparation. From the practical perspective, as it is, the method in Study II, involving the isolation of EVs with several centrifugation steps before determining EV concentration with NTA, would be difficult to translate and upscale for the needs of quality control of hundreds of samples a day. Additionally, varying information on EV storability exists, as stability in short-term storage at 4 °C or room temperature [376–378] has been indicated by some studies, while other publications suggest that EVs are unstable in these conditions and recommend either storing EV samples at -20 °C or colder [208,379] or analysing the samples immediately [380]. Another limitation is the preparation of a reliable positive control, as different activators result in different amounts of EVs from platelets [169,170] and inter-individual variation in the platelet reaction capability towards stimuli [169]. Moreover, the definition of absolute working range EV values for platelet concentrates with activated platelets would be difficult due to the heterogeneity of the platelet concentrates. Typically, buffy coats (and

[1] *Blondie*

residual plasma) from 4 donors are used to produce a single platelet concentrate, which results in varying baseline EV populations that may interact with the components of the blood product, even further activating the platelets [381,382]. Furthermore, as shown by us previously, the EV count is not a definitive indicator of an altered platelet status, as platelets can secrete EVs with different compositions while the number of secreted EVs remains unchanged [169]. Despite these limitations, several other publications indicate the value of EVs as a blood product quality control marker, and from practical perspective, the standardised determination of platelet-derived EVs with nano flow cytometry capable of detecting EVs [367], dynamic light scattering-based applications [383,384], or microfluidics [385] might offer better pipelines for high-throughput EV quantification.

Based on these factors, EV quantification as a quality marker has limitations. However, besides quantification, with the composition of platelet-derived EVs being activation-dependent, the characterisation of platelet-derived EVs could provide a better insight into the conditions platelets in the concentrate are exposed to: in this study also the size distribution of produced EVs was influenced by the storage conditions. Therefore, albeit more laborious, the compositional differences rather than the quantity of EVs should be analysed to gather more relevant information about the original cells and overall effects of the transfused unit in patients. As an example of critical information, the coagulation potential of the transfused unit could be assessed through the determination of the TF activity of EVs [386], although the TF measurement alone is problematic, and even more so when the standardised measurements of EV and TF are combined (reviewed in [387]).

Another crucial signalling pathway that EVs mediate is the lipid signalling. The time-dependent lipidome alteration of EVs was apparent at the level of phospholipid classes, and more importantly, from the perspective of LM production, at the species level. In this study, the relative amount of PS and PE in EVs was increased at the expense of PC. As LDL and HDL contain mostly PC [388], the result most likely reflects the increased EVs-to-lipoproteins ratio (Fig. 15A). The influence of the increased EVs-to-lipoproteins ratio was also observed on the GPL species level, as the relative amount of the most prominent PC species in lipoproteins, PC 34:2 [388], was decreased. However, as very little PS and PE are present in lipoproteins [388], the findings in these classes are due to the EV fraction of the concentrates. On GPL species level, the EV fraction initially contained EVs with relatively short and highly unsaturated PE, PEp, and PS, GPL species with a high efflux propensity [389], promoting their rapid exit during the early budding of EVs from the plasma membrane. Upon storage, the ageing platelets secreted EVs of a different type, which was manifested as an increase in the relative amount

of AA-containing species, especially observable in the PE and PS classes, and the respective decrease in the relative amount of GPL species with high efflux propensity. The results of the GPL examination are well in line with previous publications [264,390,391]. Finally, the phospholipid profile of EVs differed from that of platelets and platelet concentrates, indicating that certain types of lipids became enriched in EVs. Similar conclusions have also been made by others, showing enrichment of sphingolipids, PE plasmalogen and lysoPC species in EVs of platelet concentrates [392]. In the assessed aging platelet concentrate samples, the efflux propensity of GPL species most likely explains the altered GPL composition of EVs, but other mechanisms cannot be excluded. Knowing the activation-dependent (protein) composition of platelet-derived EVs, it could be extrapolated that exposure to different antagonists could influence the lipidome of EVs, indicating also the involvement of other factors, such as preference of cellular location of EV production. However, this assumption is purely speculative and deserves further assessment. Taken together, the results demonstrate the spatiotemporal secretion of EVs: the type of EVs that platelets secrete are dependent on the age, storage conditions, and activation status of platelets, however, their molecular composition is not dictated by the composition of the platelets.

One noteworthy remark on result interpretation in Study III concerns the units: as the results of Figures 12 and 13 are expressed as molar percentages, a relative measure of quantity, it does not take into account that the EV content of platelet concentrate increases during the storage, as shown by us (Studies II and III) and others [367–369]. In the study in question, the particle concentration in EV samples increased 3-fold by day 8, therefore as absolute quantities, the increase of the AA-containing species is even greater, as the relative amounts of e.g., PE 38:4 and PS 38:4 increase from 25 to 45 mol% and from 15 to 45 mol%, respectively (Fig. 13). In conclusion, notable quantities of especially AA are stored within the EV fraction for lipid-mediated reactions, either to be transferred to the target cell or to be enzymatically modified to bioactive molecules in EVs.

The compositional changes of EVs from fresh to aged platelet concentrate may translate to functional differences: in practice, the increased EV count with an enriched PS and PE profile may lead to increased procoagulant potential in platelet concentrates due to increased assembly sites for procoagulation complexes [393,394], which is an effect curiously reversed by the presence of high-density lipoproteins [395]. As stored platelet concentrates contain more activated platelets and EVs compared to fresh platelet concentrates, the procoagulant potential is increased via two different mechanisms. Also, the potential role of EVs in ATR has been speculated before [396–398],[2] supported by the observation that washing the platelets with PAS, which removes plasma and EVs from

[2] *Angel Eyes*

concentrates, results in fewer incidences of ATR [399–402], but does not dramatically influence the platelet functionality [403,404].

When the interplay of composition and functional effects of EVs is examined further, the signalling potential of EVs will be better understood. Additionally to the enriched phospholipid profile compared to original cells, EVs contained PLA_2, the enzymatic requirement for cleaving FAs from the membrane of EVs [405], and COX, LOX, and CYP enzymes for the further processing of FAs into either proinflammatory or pro-resolving LMs. In this study, the enzymatic activity was indirectly assessed from platelet concentrates proving that the majority of pathway markers are a direct result of enzymatic processing rather than oxidation, as enzymatic processing typically produces pathway markers with defined chirality [406,407], and due to auto-oxidation or hindered enzyme functionality the ratio of two different chiralities between enantiomers approaches 1:1 [408,409]. Although the enzymatic activity in EV fraction was not assessed directly, compelling evidence exists suggesting that the enzymes transported within EVs are active and capable of processing FAs [76,158–162]. The GPL composition and the presence of various enzymes indicate that EVs do not participate solely in proinflammatory or proresolution signalling, but rather represent dynamic and homeostatic means of influencing the surrounding environment.

Although more prominent in platelet concentrate samples, it could also be seen in the EV fraction that the alterations in the relative frequency of precursor FAs were not translated to changes in the produced pathway markers, for instance 12-LOX products. The observation underlines that there are preferences to what FAs the enzymes process [410], and further, this finding indicates that the regulating step in the process of producing bioactive LMs from GPLs is enzymatic activity.

More pathway markers than bioactive LMs were detected in EVs, most likely because the bioactive LMs are required only in pico to nanomolar concentrations to exert their function [324] and thus their detection in the EV fraction is technically difficult. Additional challenge in the LM detection during the storage of platelet concentrate is their short-lived presence in a bioactive form. The finding supports the role of EVs as mobile units transferring pre-processed material for further enzymatic modification when needed. Considering the EVs in plasma, and their massive lipid signalling potential, it can be concluded that EVs are an efficient and crucial extension of the lipid-mediated intercellular signalling of platelets, underlined also by the detection of LMs unique to EVs (SPMs RvE3, RvD3, MaR1). Platelet concentrates have been shown to contain residual EVs from different cellular sources, as shown by us (Table 1 in Study II) and others [411], but as the platelet concentrates are closed systems, the massive increase in LMs and pathway markers in the EV fraction can only be explained with newly secreted EVs contributing to the lipidome of the sample. Based on this study, a direct answer to the question

of what factors influence the LM and pathway marker content of EVs cannot be given, but it can be speculated that the composition is the result of the combined effect of random/specific packaging of GPLs, LM, pathway markers, and the enzymes, and on the other hand, the enzymatic activity of EVs, which could process the available GPLs, FAs, and pathway markers. Enzymatic activity within EVs has been demonstrated in cancer cell-derived EVs [412] and hepatocyte-derived EVs [413], and this could explain also the highly enriched molecular composition of EVs compared to cells, as also demonstrated by us previously [168]. In this experimental setting, the EV number increased only 3-fold, whereas the 12-HETE increased approximately 200-fold in the EV fraction. As the pathway marker accumulation is not explained purely by the increased EV number, the result more likely points towards enriched packaging and/or enzymatic activity within EVs. What factors influence enzyme loading, and more importantly, enzymatic activity within EVs and to what extent these aspects explain the molecular enrichment of platelet-derived EVs remain to be clarified by future studies.

As a limitation of LM quantification, every time point studied represents only a snapshot of the current EV population within the sample, and the dynamic interactions between cells and EVs can only be speculated. As EVs are crucial part of intercellular communication, one could also hypothesise that EVs are absorbed by other cells in the concentrate or expel their contents, which impacts the detected LM and pathway content of the EV fraction.

As at least 68% of EVs in platelet concentrates are from platelets (Table 1 in Study II), this study, together with others, confirms that platelet-derived EVs have several ways to influence the recipient cells from the perspective of lipids.[3] First of all, EVs have a specific phospholipid profile that can be incorporated into the plasma membrane [33,414] for starting material of the synthesis of bioactive LMs. Regarding LMs, EVs can transfer (unique) bioactive LMs or pathway markers to the proximity of the cells or into the recipient cell, where they are further processed. Finally, EVs contain a wide selection of active enzymes related to lipid signalling, which can be transferred to other cells, enabling even the synthesis of new types of eicosanoid-derivatives [78]. Based on this study, the mechanisms of LM-signalling cannot be explained in detail and need to be further assessed, however it has been indicated that 12-HETE, which strongly accumulated in the EV fraction, promotes the internalisation of platelet-derived EVs by neutrophils [76]. Rather than being passive transportation shuttles, it is tempting to speculate that EVs are an active extension of the intercellular signalling of platelets, "metabolic machines" capable of processing their GPLs or pathway markers into bioactive LMs, both proinflammatory and SPMs, upon need using the cargo EVs carry.

[3] *Tuco*

The lipid-mediated signalling of platelets and their EVs is a potent way to influence the surroundings [78,415]. In murine model, the foreign EVs are removed from blood circulation in a matter of minutes to the spleen, liver, lungs, and kidney [416], but it is noteworthy that RBCs and platelet-derived EVs have been shown to directly interact with e.g., monocytes [417,418]. The LMs present in platelets reinforce their role as immune cells, as proinflammatory LMs and SPMs are defined through their immune-mediating action. Indeed, platelets have also been shown to influence the surrounding cells through LM-mediated signalling [260].

As EVs are the main focus of this thesis dissertation, the lipoprotein contribution to intercellular messaging was not addressed in this study, although lipoproteins are an essential element of plasma and therefore also of transfused RBC and platelet concentrates. Besides the influence described in the results (Fig. 12 and 13), it has to be acknowledged that the lipoproteins can also interact with blood cells [419–421] and EVs [150,213], adding yet another layer of complexity to the multifaceted interactions occurring in the bloodstream.

This study, together with others, suggests that EVs of blood products can provide vital information on the cells and could be harnessed to function as part of the rigorous quality control monitoring of blood products [367–369,422]. However, EVs are even more valuable for quality control of blood products, as EV assessment would not only help to understand platelets better, but would also provide a complete picture and better overall comprehension of the whole transfused unit, including cells, EVs, and residual plasma. This study indicates that a fresh platelet concentrate on day 1 differs notably from an old platelet concentrate in terms of lipid composition, EV content, and platelet activation (Studies II and III). Still, as demonstrated by others as well, even the 5-day storage of a similarly prepared platelet concentrate fulfils the release criteria of safe blood product and does not result in a dramatic effect on platelet functionality, as measured by platelet activation (CD62P exposure), apoptosis or cell death (PS exposure), and metabolism (lactate production) [372]. However, the possibility to extend blood product storage has been studied by the means of cold-storage of platelet concentrates, improved PASs, and pathogen inactivation, resulting in promising conclusions [423–425]. If platelet concentrate storage is extended to minimise the number of platelet concentrates going to waste, the contribution of EVs to the outcome of transfusion will have to be reconsidered.

By thoroughly understanding the different components of blood products, their safety could be evaluated on a detailed level, enabling safer transfusions. From another perspective, the time-dependent tailoring of the platelet concentrate composition could be exploited by offering different kinds of concentrates for different types of patients: patients with cancer could benefit from platelet concentrates with fresh platelets that would not be removed immediately from circulation, whereas older platelet

concentrates with activated platelets ready to aggregate and possibly with procoagulant EVs might be better suited to the treatment of massive bleeding [369]. Transfusion medicine is considered to be the first example of personalised medicine, but based on this study and others, the personalisation could be taken even further by analysing and matching the EVs of blood products with the needs of the patients [369,426]. To summarise, EVs of a platelet concentrate not only provide a means of assessing platelet functionality, but also offer a way to rapidly influence their surroundings through their LM-related signalling potential. Therefore, the traceable, accurate assessment of EVs of platelet concentrates, in particular, is crucial for determining the overall effects of blood transfusions, for possibly evaluating the applicability of blood products to different transfusion needs, and ultimately for conducting safer transfusions by preventing ATR.

5.3 FUTURE PERSPECTIVES

Aside from the role of especially lipid-based EV-mediated cellular functions in platelets, to which this study also adds, it is tempting to speculate about the possibilities offered by EVs to theranostics. The concept of applying platelet-derived EVs as a marker for platelet activation is encouraging and immediately summons up the thought of extrapolating the finding to diagnostics. However, EV quantification, even from a specified cellular source, can only provide useful information in a carefully defined context, such as the quality control processes of blood products. Even if the current technical limitations, e.g., detection limits of techniques and lack of standards, hindering the accurate determination of the EV count were ignored, the application of (platelet-derived) EV number as a clinical diagnostic marker presents challenges. Determining a (patho)physiological status of patients based on the quantification of plasma EVs would be problematic, as in addition to the intra- and inter-individual variation, age, and gender [427–429], also multiple physiological conditions such as menstrual cycle and exercise [430–435] and on the other hand pathological conditions such as trauma, smoking, and physical inactivity [244,436,437] influence the EV levels of plasma. Due to these reasons, the determination of the physiological plasma EV count, similar to physiological blood cell count [238], which would serve as a vital point of comparison, is difficult. Furthermore, connecting an increased or decreased EV count to any relevant or irrelevant phenomena is problematic and even if such parameters could be developed, the inter-individual variation in the cellular response to different agonists makes it challenging to identify the correct reason for the possibly altered plasma EV counts. Therefore, it is unlikely that EV quantification even from one cellular origin in a different medical context could be used as a definitive diagnostic tool, but it could act as an indication of irregularity, which requires further studies.

However, also the findings of this study demonstrate that platelet-derived EVs have the potential for broader clinical application, as, besides the inherent structural diversity [438], the composition of platelet-derived EVs is agonist-dependent [169–172]. Presuming that these molecular indications can be reliably isolated, detected and deciphered, platelet-derived EVs have the potential to become biomarkers for different conditions, resulting in improved tools for diagnosis and prognosis. As the interplay between cancer and platelets has been demonstrated on the cellular level [439–444], it is not farfetched to consider that cancer progression could be monitored using circulating platelet-derived EVs [445–447]. Another interesting concept would be to address the relationship between cardiovascular diseases and platelet-derived EVs [448–450].

As blood products are a generous source of different kinds of nanoparticles that can be either naturally derived (EVs) or mass-produced (NanoE) from cells. Blood concentrate-derived nanoparticles could be utilised in therapeutics: one way to use EVs, especially specific subpopulations of platelet-derived EVs with desired characteristics [100], would be to exploit their natural characteristics and to apply them as natural drugs in different applications, such as wound healing [451], bleeding [28,114,452] or to modulate immunological reactions based on their lipid mediator content [76,159,453–457].

Another alternative is to chemically engineer EVs or other blood product-derived semisynthetic nanoparticles, such as NanoEs, to contain preferred therapeutic components [195,298,458–461] and use them as a drug delivery system. A particularly interesting feature of EVs is their ability to pass the blood-brain barrier [462–465], indicating that EVs have potential as drug delivery systems in the treatment of different neurological diseases. The EV targeting and uptake is not yet completely understood as preferential cellular uptake of EVs from the same cellular source has been demonstrated [466], but also evidence of non-selective incorporation of EVs to recipient cells using cell-type specific uptake mechanisms exists [467]. Our own unpublished results demonstrate a differential uptake of RBC and platelet-derived EVs into cancer cells, which indicates that certain types of EVs may contain molecular characteristics facilitating the cellular uptake process. It is important to carefully analyse the composition, and particularly the lipid composition, of different populations of EVs to determine how the compositional differences influence the uptake and whether such entities could be replicated in semisynthetic alternatives, such as NanoEs or liposomes [468]. NanoEs have been used in drug delivery before [469,470], and supportive evidence for the use of semisynthetic drug delivery systems over EVs shows that natural EVs could have counterproductive effects, as especially blood product-derived EVs may enhance pro-inflammatory responses [117,471] and coagulation [28,114].

6 FINAL CONCLUSIONS

The details of EV functionality still remain to be determined and to fully comprehend these curious little particles, methods and measurements in the rapidly developing, highly method-dependent field of EVs need to be standardised. To improve the comparability of measurements, a biological reference material similar to EVs and applicable to multiple EV quantification and characterisation techniques was produced and confirmed to be similar to naturally produced EVs in many aspects. Through the transparency and comparability of results, the role of EVs in a physiological and pathophysiological context can be understood better, which leads to the possibility of capitalising the knowledge of EVs in the development of different applications: in the very specific context of platelet concentrates, EV quantification already provides substantial information about the platelets, as platelet concentrate EVs might be useful as a marker of platelet activation.

Besides preventing anaemia and maintaining haemostasis, RBCs and especially platelets have more complex functions than initially appears, as these cells actively participate in the modulation of immune reactions in yet poorly understood ways. As EVs are shown to facilitate and even mediate these reactions, the accurate assessment of EVs and their molecular composition, especially in lipid signalling, in relation to the cell functions was the focal point of this thesis work. This thesis demonstrates that lipid-mediated signalling in platelets is a complex entity carefully regulated by enzymatic activity. As also EVs met the molecular requirements for lipid-mediated signalling, they can be regarded as an integral part of intercellular signalling of platelets able to transfer pre-processed lipid frames and active enzymes to cells. More interestingly, EVs may even be active participants in lipid-mediated signalling capable of reacting to different conditions by producing bioactive LMs locally when needed.

Several interesting questions remain for future examination, for instance, regarding EV-mediated lipid signalling, it is critical to dissect whether certain types of LMs and pathway markers are associated specifically with different subpopulations of EVs and to determine the specific mode of action of EVs in lipid signalling (active participation or passive transporter of material). The role of particular LMs and pathway markers, e.g., such that do not show time-dependent accumulation in the EV fraction, and LMs unique to EVs (SPMs RvE3, RvD3, MaR1), and on the other hand the mechanism regulating enzymatic activity of PLA, COX, LOX, and CYP enzymes, deserve more attention in future as they could mediate ATR in platelet concentrates.

The vast molecular capacity of EVs to influence surroundings is the basis for their (patho)physiological effects, therefore the mechanisms involving EV-mediated intercellular signalling need to be understood thoroughly, which requires standardised measurements and protocols. When the EV-mediated cellular functions are understood better, novel aspects of EV assessment may require new types of techniques, possibly requiring novel method development. When the relationship between EV composition and functionality is understood better, this knowledge can be exploited to develop valuable tools for diagnostics and therapeutics from EVs or to develop other similar semisynthetic options for e.g., drug delivery based on the critical properties of EVs. However, the methodology for e.g., current drug delivery systems may not be applicable to EVs, therefore the methodology behind EV-based applications may also require some development. Then the EVs, either natural or chemically modified, or semisynthetic EV-like derivatives could provide a means of influencing cellular functionality (in e.g., cancer treatment or wound healing) or of assessing the cellular status of an individual by exploiting EVs as a valuable biomarker for different conditions (Fig. 18).

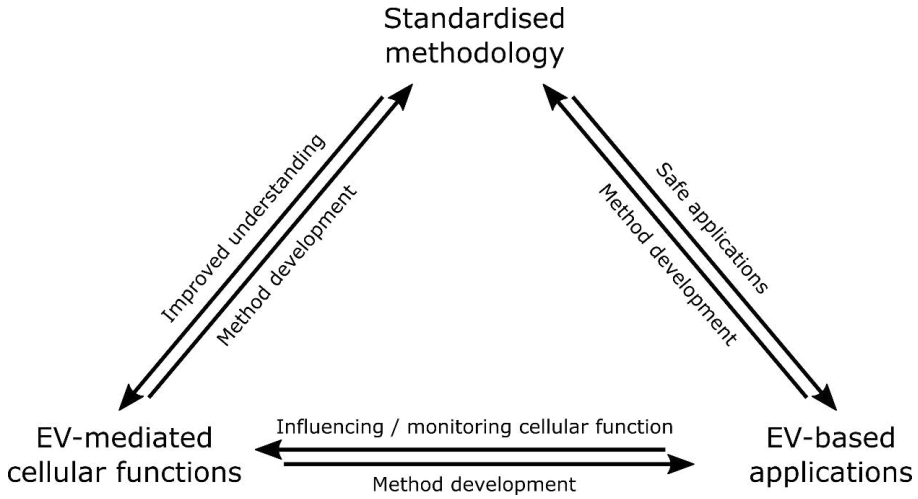

Figure 18: The three cornerstones of EV studies and their intertwined relationship, a prerequisite for the translation of EVs from bench to bedside.

To summarise, besides their biological significance in intercellular messaging, blood product EVs have enormous applicability in quality management of blood products. Building our understanding of EV-mediated functions using blood product EVs as a model could ultimately lead to the active incorporation of EVs into the clinical context as EV-based diagnostics or even therapeutics. When the added value of NanoE as reference material and potential as a drug delivery system is included, it can be concluded that blood products contain hidden treasures, pearls, in the form of these nanoparticles.

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Sami Valkonen
Helsinki, December 2019

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9 APPENDICES

Valkonen S, van der Pol E, Böing A, Yuana Y, Yliperttula M, Nieuwland R, Laitinen S, Siljander PRM. Biological reference materials for extracellular vesicle studies. Eur J Pharm Sci 2017;98:4–16.

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Biological reference materials for extracellular vesicle studies

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ABSTRACT

Extracellular vesicles (EVs) mediate normal physiological homeostasis and pathological processes by facilitating intercellular communication. Research of EVs in basic science and clinical settings requires both methodological standardization and development of reference materials (RM). Here, we show insights and results of biological RM development for EV studies. We used a three-step approach to find and develop a biological RM. First, a literature search was done to find candidates for biological RMs. Second, a questionnaire was sent to EV researchers querying the preferences for RM and their use. Third, a biological RM was selected, developed, characterized, and evaluated.

The responses to the survey demonstrated a clear and recognized need for RM optimized for the calibration of EV measurements. Based on the literature, naturally occurring and produced biological RM, such as virus particles and liposomes, were proposed as RM. However, none of these candidate RMs have properties completely matching those of EVs, such as size and refractive index distribution. Therefore, we evaluated the use of nanoerythrosomes (NanoE), vesicles produced from erythrocytes, as a potential biological RM. The strength of NanoE is their resemblance to EVs. Compared to the erythrocyte-derived EVs (eryEVs), NanoE have similar morphology, a similar refractive index (1.37), larger diameter (70% of the NanoE are over 200 nm), and increased positive staining for CD235a and lipids (Di-8-ANEPPS) (58% and 67% in NanoE vs. 21% and 45% in eryEVs, respectively).

Altogether, our results highlight the general need to develop and validate new RM with similar physical and biochemical properties as EVs to standardize EV measurements between instruments and laboratories.

1. Introduction

Extracellular vesicles (EVs) are lipid bilayer surrounded particles that contain proteins, lipids, metabolites, and nucleic acids (Yanez-Mo et al., 2015). EVs are produced by most cells, including bacteria and plant cells, making cross-kingdom communication possible (Samuel et al., 2015). EVs have active physiological and pathophysiological roles and they are functional components of intercellular communication, thereby offering possibilities in the development of therapy and diagnostics, or collectively, theranostics (Fais et al., 2016). EVs are often classified into exosomes and microvesicles based on size and the route of formation, but increasing data have revealed this to be an oversimplification, since the isolated populations are heterogeneous and have overlapping properties including size, density, and molecular markers (van der Pol et al., 2016).

The molecular content and concentrations of EVs in human body fluids have raised increasing interest for their use as biomarkers (Fais et al., 2016). A biomarker based on EVs has not yet been realized, partly due to the lack of standardization. Standardization is difficult because the calibration of instruments, the interpretation and validation of results, and the comparison of measurements require a reference material (RM) with physical properties equal to EVs. One of the most analyzed property of an EV sample is the concentration. However, the measured EV concentration depends on the physical properties of EVs, such as the size distribution and refractive index (RI), complicating the analysis, as explained below.

EVs smaller than 300 nm constitute the majority of EV population (Aatonen et al., 2014; Arraud et al., 2014; Dragovic et al., 2011, 2013; Gercel-Taylor et al., 2012; Varga et al., 2014; Yoshioka et al., 2013). Typical size distributions of EVs start at ~30 nm, show a peak at a

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diameter < 100 nm, and follow a decreasing power-law function or exponential function for diameters >100 nm (Fraikin et al., 2011; van der Pol et al., 2016). With the exception of transmission electron microscopy (TEM), none of the current analytical methods are able to detect the entire population of EVs (van der Pol et al., 2016). The inability to detect the smallest EVs leads to both differences and underestimation of the determined concentration. Consequently, the reported number of EVs in normal human plasma ranges from 10^4 to 10^{12} mL^{-1} (van der Pol et al., 2014a). This 8 orders of magnitude difference in EV concentrations emphasizes the need for standardization.

In flow cytometry, which is one of the most commonly used methods in EV studies (Lacroix et al., 2010), particle detection is often based on light scattering. Because the RI of silica (1.45) and polystyrene beads (1.61) is higher than the mean RI of naturally occurring EVs (~ 1.39), applying a gate on the scatter signals of silica or polystyrene beads will result in erroneous estimations of EV size and concentration (van der Pol et al., 2012, 2014b). For example, a lower size gate set with 200 nm polystyrene beads, which scatter the same amount of light as EVs of ~500 nm (Chandler et al., 2011), leads to the exclusion of EVs between 200 and 500 nm (van der Pol et al., 2014b). Since the concentration of EVs decreases with increasing diameter, a polystyrene size gate generally leads to an underestimation of the actual EV concentration.

With nanoparticle tracking analysis (NTA) the Stokes–Einstein equation is used to derive the hydrodynamic diameter of EVs from their Brownian motion (Dragovic et al., 2011). Although in NTA, the RI of EVs does not affect the measured diameter, the EV size distribution and RI do affect the measured concentration (Filipe et al., 2010), because the measured concentration depends on the brightness of the scattering particle.

Altogether, these examples emphasize the urgent need to develop RM with a similar RI and size distribution, but preferably also with a morphology (for TEM) and zeta potential (for tunable resistive pulse sensing, TRPS) similar to the studied EVs. Ultimately, also other RM properties would match those of EVs, including surface molecules or internal cargo. This is challenging because the development of an optimal RM for EV studies and the analytical methods for their detection are dependent on each other. Further, the different analytical techniques depend on different properties of EVs (Table 1). In this study, we took a three-step approach to develop RM for EV studies: a literature search was performed to find candidates for biological RM, and then EV researchers were asked for the preferences for RM and their use. Finally, we took a step forward and developed an erythrocyte-derived EV-RM, nanoerythrosomes (NanoE), and evaluated its usability.

2. Materials and Methods

2.1. Literature Search

The task of discovering a potential biological RM for EV studies was initiated through discussions with various professionals of "Metrological characterization of micro-vesicles from body fluids" (METVES; www.metves.eu) program. Based on the discussions, the initial categories of RM were determined and a literature search was conducted in Google and PubMed using terms such as "erythrocyte ghost", "RBC carrier", "outer membrane vesicles", "nanobacteria", "viral particle", "coccoid bacteria", "liposome", "cell organelle", "stability", production", and "preparation" to elaborate the properties of potential RM. Initial inclusion criteria for potential RM were submicron size and organic composition. To further investigate the benefits of the selected RM, experts from the EV field were consulted regarding the properties of potential RM from the literature search. Candidates were excluded if the particles contained infection risk, did not express sufficient physical and biochemical resemblance to EVs, or were poorly storable. The literature search and expert consultation was conducted from 10/2014 to 11/2014.

Table 1
Dependency of the different detection techniques on EV properties and EV sample properties.

	AFM	DLS	FCM	NTA	SAXS	TEM	TRPS
Adhesion	+	−	−	−	−	+	−
Buoyancy	−	−	−	−	−	−	±
Charge	−	±	−	±	−	−	+
Concentration	+	+	+	+	++	+	+
Membrane proteins	±	−	±	±	−	±	−
Monodispersity	−	++	−	±	++	−	−
Refractive index	−	+	++	+	−	−	−
Size	−	+	+	+	++	−	+
Spherical shape	−	++	++	+	+	−	++
Stiffness	+	−	−	−	−	+	−

Abbreviations: AFM: atomic force microscopy; DLS: dynamic light scattering without charge option; FCM: flow cytometry; NTA: nanoparticle tracking analysis without charge option; SAXS: small-angle X-ray scattering; TEM: transmission electron microscopy; TRPS: tunable resistive pulse sensing.

2.2. Survey of RM and Their Use in EV Studies

A questionnaire (Appendix 1) was designed to collect the following information: methods in use for the characterization and quantification of EVs, current use of RM, desired and minimal physical and biochemical requirements of RM, and opinions of other potential RM. The questionnaire was sent to 14 stakeholders from the METVES program and 32 collaborators from the Laboratory of Experimental Clinical Chemistry (Academic Medical Center, Amsterdam, Netherlands) working with EVs. Replies were collected from 11/2014 to 12/2014.

2.3. Preparation of RM from Erythrocyte Concentrates

Standard leukocyte-reduced erythrocyte concentrates were used to produce NanoE. Outdated concentrates were obtained from Sanquin (Amsterdam, The Netherlands) and the Finnish Red Cross Blood Service (Helsinki, Finland). Concentrates were handled anonymously, and only concentrates that could not be administered clinically were used as accepted by the Finnish Supervisory Authority for Welfare and Health (Valvira, Finland).

To isolate erythrocyte-derived EVs (eryEVs), 25 mL of the concentrate was diluted with 25 mL of 0.22 μm filtered calcium- and magnesium-free 1× phosphate-buffered saline (PBS [Sigma-Aldrich, St. Louis, MO, USA]) and centrifuged for 20 min at 1560×*g*, room temperature (RT) without brake (Centrifuge 5810 R, Eppendorf, Hamburg, Germany) (Varga et al., 2014). Supernatant was transferred to new tubes and centrifuged 3 times under the same settings. The obtained supernatant was centrifuged for 1 h in 100,000×*g* at 4 °C (Optima™ MAX-XP Ultracentrifuge with rotor TLA-55, k-factor 66, Beckman Coulter, Brea, CA, USA), after which the pellet was washed with similar ultracentrifugation. Finally, the pellet was suspended with PBS to the initial volume and aliquoted to 100-μL aliquots for storage at − 70 °C (Fig. 1A).

NanoE production was initiated by separating the erythrocytes from the concentrate: 25 mL of concentrate was diluted with 25 mL cold (+ 4 °C) PBS and centrifuged at 300×*g* for 10 min at 4 °C without a brake (Centrifuge 5810 R). The pellet was suspended to an equal volume of cold PBS, centrifuged 1560×*g* for 20 min at 4 °C without a brake (Centrifuge 5810 R). The washing was repeated 2 more times. Next, three different disruption methods were evaluated to produce NanoE:

Freeze-thawing: 500 μL aliquots of erythrocytes were treated with 3 consecutive freeze–thaw cycles of 5 min in liquid nitrogen and 5 min in 37 °C water bath.

N_2 bomb treatment: 5 mL of erythrocytes were diluted with 10 mL of PBS in 50 mL Falcon tube to facilitate nitrogen access to the cells. The tubes were placed in N_2 bomb (Parr Cell Disruption Bomb, Moline, IL, USA). A pressure of 75 Psi was created using nitrogen and after

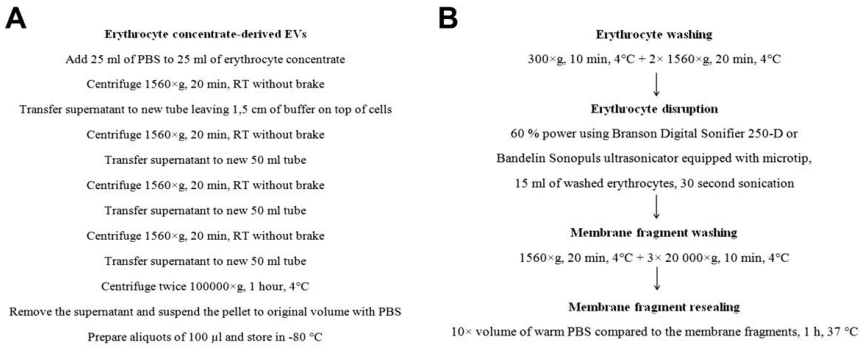

Fig. 1. A step-by-step protocol for harvesting EVs from erythrocyte concentrate (A) and schematic of the production of nanoerythrosomes (B).

30 min, the pressure was gently released and the sample was collected in a 50 mL Falcon tube.

Ultrasonication: 15 mL of erythrocytes were sonicated in an ice bath with either Branson digital sonicator 250-D (Branson Ultrasonics, Danbury, CT, USA) equipped with microtip using continuous sonication, 60% power and 30 or 45 s sonication, or Bandelin Sonopuls ultrasonicator (BANDELIN electronic GmbH & Co. KG, Berlin, Germany) equipped with MS73 microtip and 30 s continuous ultrasonication.

After disruption, the suspensions containing membrane fragments were diluted with an equal volume of cold PBS, and centrifuged for 1560×*g* at 20 min and +4 °C without brake (Centrifuge 5810 R) to remove remaining cells and larger fragments. Remnants were washed by transferring 500 µL aliquots of supernatant to Eppendorf tubes, diluting the suspension 1:1 with cold PBS and centrifuging 10 min in 20,000×*g* for 10 min and +4 °C without brake (Mikro 200R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Remnants were washed 3 times by suspending the pellet in 1 mL of cold PBS. The washed pellet was suspended in 100 µL of +37 °C PBS and transferred into 10× volume of +37 °C PBS to allow the resealing process at +37 °C (water bath) for 1 h. After the resealing process, the NanoE were aliquoted to 100 µL aliquots and stored in −70 °C.

2.4. Transmission Electron Microscopy

Samples were fixed 30 min in 0.1% (weight/volume, *w*/*v*) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Next, a 200-mesh EM copper grid with formvar coating (Electron Microscopy Sciences) was placed on top of a sample (10 µL), and incubated for 7 min at RT. The grids were transferred to 1.75% uranyl acetate (*w*/*v*) for negative staining. The grid was imaged using a Tecnai 12 transmission electron microscopy (TEM, FEI Company, Eindhoven, The Netherlands), operated at 80 kV.

2.5. Nanoparticle Tracking Analysis

The same NanoE samples were measured with the instruments LM14C, NS300, and NS500 using the same settings (camera level 8, 3 videos of 90 s, 10,000-fold dilution). Analysis of the acquired videos was performed with threshold 5 and gain 10. The used NTA instruments and their specifications are listed in Table 2. LM14C was also used to study how storage affects the size distribution and the concentration of NanoE. NanoE samples were first measured with NTA immediately after preparation and then up to 10 weeks with biweekly measurements (Supplementary Fig. 1).

2.6. Flow Cytometry

NanoE and eryEVs were characterized using Apogee A50 micro (Apogee Flow Systems, Hertfordshire, UK) equipped with a 405 nm laser for measuring scatter and a 488 nm laser for measuring fluorescence. Fluorescence light was spectrally filtered by bandpass filters (525/50, 575/30), and a long pass filter (650 nm). Particles were labeled with a FITC-labeled anti-CD235a antibody (clone 11EB-7-6, Beckman Coulter, Brea, CA, USA) and a lipid dye Di-8-ANEPPS (Invitrogen, Waltham, MA, USA). For CD235a, the NanoE and eryEVs concentration was ~10^8 mL^{-1} as determined by NTA (LM14C). The labeling volume was 100 µL. Possible antibody aggregates were removed before use by centrifuging for 5 min at 18,890×*g* and 20 °C without brakes, and 10 µL of the antibody was used for each sample. After 30 min incubation in the dark, the labeling reaction was stopped by adding 900 µL of 0.22 µm filtered PBS. IgG1-FITC was used as an isotype control (clone MOPC-21, BD Pharmingen, San Jose, CA, USA). For the Di-8-ANEPPS labeling, 1 µL of 12.5× Di-8-ANEPPS lipid dye preparation (1 µL Di-8-ANEPPS, 6.25 µL pluronic acid (product code #P3000MP [Thermo Fisher, Waltham, MA, USA)], and 5.25 µL mQ water) was added to 200 µL samples of eryEVs and NanoE at a concentration of ~10^7 mL^{-1}. As free Di-8-ANEPPS aggregates in the buffer, the amount of Di-8-ANEPPS-positive particles in the buffer without EVs was deducted from samples in data analysis. Samples were incubated for at least 30 min, RT, covered from light. For sample detection, large-angle light scattering or small-angle light scattering was used as a trigger and used voltages and thresholds were 320 and 31 for large-angle light scattering and 295 and 14 for small-angle light scattering, respectively. Samples were injected at 4.5 µL/min, data was collected for 120 s for each sample and three washing cycles were performed between the samples. NanoE and eryEVs were additionally compared to bead mixture of silica and polystyrene beads (Apogee Flow Systems).

2.7. SDS-PAGE

The protein compositions of eryEV and NanoE samples were studied by loading equal amounts of 0.3 µg of protein (determined with µBCA kit [Thermo Fisher Scientific]) together with Multicolor broad range protein ladder (Thermo Fisher Scientific) to commercial Mini-Protean TGX 10% gels (BioRad, Hercules, CA, USA). The gels were run with 180 V for 1 h in 1× Tris/glycine/SDS buffer (BioRad). The gel was fixated (30% ethanol, 0.5% acetic acid [Merck, Kenilworth, NJ, USA]) for 1 h, after which it was rinsed for 10 min in 20% ethanol and 10 min in water. The gel was sensitized using freshly prepared sodium thiosulphate (0.02 g/100 mL [Sigma-Aldrich]) for 1 min. The gel was rinsed twice in water for 20 s and stained in freshly prepared silver nitrate (0.1 g/50 mL

Table 2
Specifications of the NTA instruments and software.

NTA instrument	Laser	Camera	Software version in data collection	Software version in data analysis
LM14C	Violet laser: 405 nm, 70 mW (Malvern Instruments Ltd., Malvern, UK)	sCMOS camera (Hamamatsu Photonics K.K., Hamamatsu, Japan)	3.0	3.1
NS300	Violet laser: 405 nm, 65 mW (Malvern Instruments Ltd.)	sCMOS camera (Hamamatsu Photonics K.K.)	3.1	3.1
NS500	Violet laser: 405 nm, 45 mW (Malvern Instruments Ltd.)	EMCCD camera (Andor Technology, Tokyo, Japan)	3.1	3.1

[Merck]) for 30 min. The gel was rinsed in water for 10 s, after which it was developed using freshly prepared development solution (70 μL of 37% formaldehyde [Merck], 3 g of potassium carbonate [Sigma-Aldrich], 1 mg of sodium thiosulphate [Sigma-Aldrich], and added to 100 mL of water) for 4 min. The development was stopped with incubation in stopping solution (50 g/L Tris base [Merck], 2.5% acetic acid [Merck]) for 1 min, after which the gel was stored in water. The Western blots were prepared as mentioned before (Aatonen et al., 2014).

2.8. Determination of Refractive Index

The RI of NanoE and eryEVs were determined by independently measuring the diameter and the light scattering power of individual particles with NTA and solving the inverse scattering problem with Mie theory (van der Pol et al., 2014b).

2.9. Statistics

Statistical significance was determined by using two-tailed *t*-test (GraphPad Prism v.5.0.1.)

3. Results

3.1. Literature Search

Although several studies have characterized and described the use of monodisperse (Lacroix et al., 2010; Chandler et al., 2011; Maas et al., 2015) and bimodal (Nicolet et al., 2016) synthetic RM, the reported use of biological RM is limited (van der Pol et al., 2012; Anon., n.d.-a). The following literature search describes potential, naturally occurring or "produced" RM, which could be further developed for EV analyses.

3.1.1. Naturally Occurring Sources for Biological RM

Submicron particles with physical and biochemical properties similar to EVs can be isolated from naturally occurring sources. These may include 1) isolated EV populations from, e.g. cell cultures, 2) plasma lipoproteins, plant and marine viruses, and 3) small spherically shaped (coccoid) bacteria, or picoplankton (Table 3).

(1) Potential EV sources are *in vitro* cell cultures (Lazaro-Ibanez et al., 2014), cultures of *Dictyostelium discoideum* (Tatischeff et al., 2012), therapeutic clinical grade erythrocyte (Varga et al., 2014) and platelet (Black et al., 2015) concentrates, urine (van der Pol et al., 2014a), and outer membrane vesicles produced by bacteria (Biller et al., 2014). Here, the specific advantage is that the obtained RM have enhanced physical and biochemical similarities, including the molecular contents, with actual EVs. These EV sources are also fairly accessible and safe. Thus, well-characterized EVs would also be the perfect EV-RM candidates.

(2) Lipoproteins and viral particles from plant and marine sources are suitable as EV-RM because they have a size distribution overlapping with the bulk of EVs (Aatonen et al., 2014; Arraud et al., 2014; Dragovic et al., 2011; Dragovic et al., 2013; Gercel-Taylor et al., 2012; Varga et al., 2014; Yoshioka et al., 2013; Anon., n.d.-b, n.d.-c; Oster, 1950; van Antwerpen et al., 1999; Sawle et al., 2002; McFarlane et al., 2005) and they do have a relatively small variation in size (van Antwerpen et al., 1999; Sawle et al., 2002; McFarlane et al., 2005; Salpeter and Zilversmit, 1968; Colhoun et al., 2002). However, a major drawback of lipoproteins and viral particles is that the RI of these particles is higher than the RI of EVs, due to their high protein content, a prominent problem especially of protein-enveloped viruses. Another issue of using viral particles is their biosafety, which could be circumvented by producing virus-like particles, i.e. particles lacking the viral genome. The mass production of virus-like particles is possible in plant or insect cells (Machida and Imataka, 2015; Santi et al., 2006).

(3) Another possible source of biological RM are non-pathogenic bacteria and picophytoplankton, i.e. aquatic organisms of both prokaryotic and eukaryotic origin ranging between 600 nm and 2000 nm in diameter (Anon., n.d.-d). Several non-pathogenic marine bacteria and picophytoplankton strains exist (personal communication with representatives of Roscoff Culture Collection; Roscoff, France) and can be purchased for culturing. The benefit of cultures of non-pathogenic bacteria and picophytoplankton is that the cultures could be harnessed into mass production to provide two populations of particles with different size distributions. Bacteria can be used as larger particles (>600 nm) and the corresponding bacteria-derived outer membrane vesicles can be used as smaller particles (<250 nm) (Biller et al., 2014). As outer membrane vesicles have comparable physical and biochemical properties as the bacteria (Biller et al., 2014), the main difference would be their size. By maintaining cultures, the biological RM would be essentially self-generating with affordable and effortless maintenance depending of the used strain. A literature search for non-pathogenic bacteria species, which could be used as biological RM based on their size, suggested several spherically shaped (coccoid) bacteria with reported diameters of <1000 nm (Bae et al., 1972; Balkwill and Casida, 1973; Barbier et al., 1999; Lai et al., 2000; Osburn and Amend, 2011).

3.1.2. Production of Biological RM

Besides harvesting EVs as RM from naturally occurring sources, submicron particles with EV-like properties can be produced from various sources. Here, we included particles produced only from biological materials including disrupted cells and different lipid constructs (Table 4).

Biological RM can be produced by disrupting cells to produce small vesicles from the fragments yielding particles with varying diameters (Marchesi and Palade, 1967; Heidrich and Leutner, 1974; Lin and Macey, 1978; Yoon et al., 2015; Jo et al., 2014). The main advantage of using such materials is that the physical and biochemical properties of the obtained RM would better resemble EVs compared to synthetic RM (Yoon et al., 2015; Jo et al., 2014). Erythrocytes are theoretically

Table 3
Naturally occurring potential biological reference materials (RM).

RM	Diameter (nm)	Polydispersity (CV)	RI	Resemblance to EVs	Considerations	References
EVs from						
Cell lines	30–1000	>20%	~1.38	5	EVs stable for months when stored −80 °C	Lazaro-Ibanez et al. (2014)
Dictyostelium discoideum	50–300	35%–70%	–	5	–	Tatischeff et al. (2012)
Erythrocyte or platelet concentrates	10–350	>20%	–	4/5	EVs stable for months when stored −80 °C	Varga et al. (2014), Black et al. (2015)
Lyophilized exosomes*	30–100	>20%	1.37–1.39	5	Commercially available, can be stored for months	Anon. (n.d.-e)
Outer membrane vesicles from (marine) bacteria	10–350	–	–	5	–	Biller et al. (2014), Li et al. (1998), Kadurugamuwa and Beveridge (1997), Schooling and Beveridge (2006), Beveridge (1999)
Urine	45–500	35%–40%	1.37	5	EVs stable for months when stored −80 °C	van der Pol et al. (2014a, 2014b), Tatischeff et al. (2012)
Lipoproteins						
High-density lipoproteins	6–15	~5%	1.45 − 1.6	4	Must be stored under nitrogen or argon, if stored in +4 °C. Can be stored at −80 °C for months	van der Pol et al. (2014b), van Antwerpen et al. (1999), Sawle et al. (2002), McFarlane et al. (2005), Salpeter and Zilversmit (1968), Colhoun et al. (2002), Perusse et al. (2001), Wood et al. (2006)
Low-density lipoproteins	18–25	<10%				
Intermediate-density lipoproteins	30	~1.5%				
Very low-density lipoproteins	30–80	~20%				
Chylomicrons	200–600	>20%				
Viral particles from						
Marine virus species	110–130	<20%	–	2	Can be stored for months in − 80 °C. No safety restrictions, if particles do not contain genomic material	Anon. (n.d.-d)
Plant virus species	20–85	<20%	1.52–1.57	2		van der Pol et al. (2014b), Anon. (n.d.-b, n.d.-c) Oster (1950)
Coccoid-shaped organisms						
Aquatic bacteria/pico(phyto)plankton	300-1000	–	1.35-1.47**	5	Can be stored for months in − 80 °C. No safety restrictions, if particles do not contain genomic material.	Biller et al. (2014), Anon. (n.d.-d), Lai et al. (2000), Ackleson and Spinrad (1988), Spinrad and Brown (1986), Kuo et al. (2014), Hashemi et al. (2011)
Nanobacteria from soil	200–1000	–	–	5		Bae et al. (1972), Balkwill and Casida (1973)

RM were categorized according to size, polydispersity, refractive index (RI), and the resemblance to the EVs. Resemblance to EVs was scored on 1–5 points depending on whether the RM has (1) no resemblance, (2) proteins and genomic material but no lipid membrane, (3) phospholipid membrane, (4) phospholipid membrane containing proteins, or (5) phospholipid membrane containing proteins and genomic material. * = Exosomes from HansaBiomed, Tallinn, Estonia. ** = Values based on the references and the refractive index of water at 488 nm.

ideal materials to use, since their cell membrane composition is well-characterized and they are devoid of intracellular membranes (Virtanen et al., 1998). Additionally, erythrocytes are structurally stable during extended storage (Chaplin, 1982) and are an easily accessible material. For these reasons, erythrocytes from clinical surplus concentrates were used to produce an RM for testing and evaluation. Different

Table 4
Produced biological reference materials (RM). RM were categorized according to size, polydispersity, refractive index (RI), and the resemblance to the EVs.

RM	Diameter (nm)	Polydispersity (CV)	RI	Resemblance to EVs	Considerations	References
Disrupted cells						
Preparation method A	<200	>20%	–	4	–	Marchesi and Palade (1967)
Preparation method B	200–700	>20%	–	4	–	Heidrich and Leutner (1974)
Preparation method C	100–5000	>20%	–	4	–	Lin and Macey (1978)
Preparation method D	100–300	20%–35%	–	5	–	Yoon et al. (2015)
Preparation method E	100	–	–	5	–	Jo et al. (2014)
Lipid constructs						
Liposomes	100	~5%	1.363–1.392	3	–	Lapinski et al. (2007), Matsuzaki et al. (2000)
Liposomes (commercially available*)	100 and 500	<20% for smaller particles, >20% for bigger particles	Varies with used buffer	3-5	Can be stored at least 12 months	Anon., (n.d.-f)
Lipoparticles**	191	13%	–	3-4	Can be stored for 18 months	Anon. (n.d.-g)
Oil droplets***	<100	<20%	Engineered to preferred RI	1	Can be stored at least 12 months	Anon. (n.d.-h, n.d.-i),

Resemblance to EVs was scored from 1 to 5 points depending on whether the RM has (1) no resemblance, (2) proteins and genomic material but no lipid membrane, (3) phospholipid membrane, (4) phospholipid membrane containing proteins, or (5) phospholipid membrane containing proteins and genomic material. * = Liposomes from Excytex, Zeist, The Netherlands; ** = lipoparticles from Integral Molecular, Philadelphia, PA, USA; *** = oil from Apogee Flow Systems, Hertfordshire, UK or Cargille Laboratories, Cedar Grove, NJ, USA.

methods for erythrocyte disruption were compared, and a method for NanoE production was developed (Fig. 1B). An additional advantage of using erythrocytes is that they can be used to produce two types of biological RM for comparison: 1) by disrupting the erythrocytes NanoE are formed, and 2) by harvesting the spontaneously shed eryEVs.

Biological RM can also be obtained from lipid constructs such as liposomes that are extensively used as delivery vehicles (Allen and Cullis, 2004; van der Meel et al., 2014) and their production methods are well known. Liposomes of a desired size can be prepared by ultrasonication or extrusion of the starting material through polycarbonate filters of a set pore size (Lapinski et al., 2007; Akbarzadeh et al., 2013). As the liposomes are produced from bulk material, their composition is well characterized. Especially, liposomes with a small diameter (~70 nm) prepared by extrusion have <20% variation in size (CV, ratio of the standard deviation to the mean, expressed as percentage) (Garcia-Diez et al., 2016). An advantage of liposomes is that their RI can be manipulated during the production (Fenzl et al., 2015). Another type of RM resembling liposomes are non-infectious virus-like particles called lipoparticles, which consist of a lipid membrane constructed on top of a protein core. These lipoparticles have a well-defined diameter of ~190 nm with a narrow size distribution (CV = 13%), and they are stable during storage. On request, additional proteins could be attached to the lipoparticle surface, which will increase their biochemical similarity to EVs.

Finally, as a non-biological exception, we include oil droplets as one option of a produced RM because they would offer close similarity with EVs regarding the size and RI. As with liposomes, extrusion can be used to prepare oil droplets of a specific size from oils of commercial providers. The advantage of using oil droplets is that the RI can be designed exactly, thus improving their resemblance to EVs (personal communication with Oliver Kenyon, CEO of Apogee Flow Systems, Hertfordshire, UK and representatives of Cargille Laboratories, Cedar Grove, NJ, USA).

3.2. Survey

The literature search revealed several different types of RM, which had varying benefits and drawbacks. Therefore, the opinions of laboratories working with EVs were surveyed through a questionnaire to discover the requirements of biological RM by the end-users. A questionnaire (Appendix 1) was sent to 46 laboratories and the response rate was 44%. Flow cytometry was indicated as the most common method for EV studies (90% of the responders; Fig. 2A),

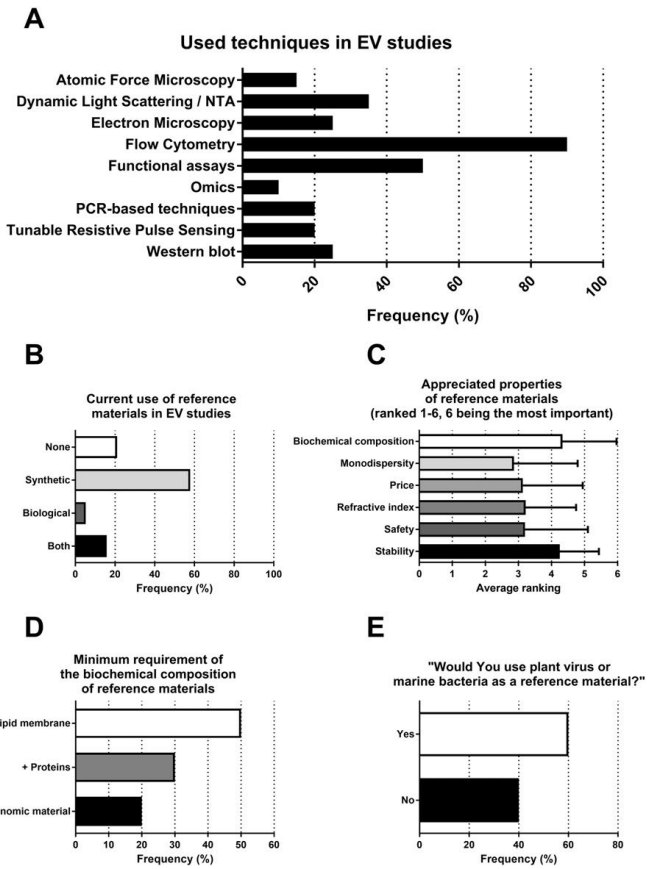

Fig. 2. Results from 46 EV laboratories based on a questionnaire asking (A) which techniques were in use for EV studies (multiple choices allowed); (B) currently used RM; (C) preferred properties for optimal RM; (D) minimal requirements for a RM; and (E) willingness to use plant viruses/marine bacteria as RM. Values represent mean ± S.D. Published previously (in www.metves.eu), reproduced with permission. NTA = nanoparticle tracking analysis; PCR = polymerase chain reaction.

underlining the need for a RM with a size and RI distribution resembling EVs. Besides using functional assays (50%), dynamic light scattering/NTA (35%), electron microscopy (25%), and Western blotting (25%) were listed as the most commonly used techniques for EV studies (Fig. 2A). When laboratories were asked whether they have used an RM in their studies, the majority reported using synthetic RM (58%), 5% used biological RM, and 16% used both. However, 20% used no RM in their EV studies (Fig. 2B).

The laboratories were asked to rank the order of importance regarding the desired properties, i.e. the biochemical composition, monodispersity, price, refractive index, and safety. The biochemical composition (average rank of 4.33/6) and stability (average rank of 4.25/6) were indicated as the most important properties (Fig. 2C). Next, price, RI, and safety were listed with an almost equal importance (average ranks of 3.13/6, 3.21/6, and 3.20/6, respectively), whilst monodispersity was considered as the least important property (average rank of 2.90/6) (Fig. 2C). Regarding the requirements for the structural properties, 50% of the laboratories working with EVs would be satisfied if the RM would contain a phospholipid membrane, 30% would additionally require proteins, and 20% would require a phospholipid membrane and the presence of both proteins and genomic material (Fig. 2D). Finally, 60% would be willing to use plant viruses and marine bacteria as RM, provided that their biosafety can be assured (Fig. 2E).

3.3. NanoE Production

NanoE were selected as a biological RM candidate, based on the literature review and the survey responses. Three erythrocyte disruption methods (freeze–thaw cycles, N_2 bomb, and ultrasonication) were tested in the NanoE production. Freeze–thaw cycles did not break down the erythrocytes sufficiently, as seen in TEM micrographs (Fig. 3A), although the treatment made the erythrocytes leaky, resulting in white erythrocyte ghosts. Furthermore, the number of submicron particles was almost non-existent (Fig. 3B). Disruption using an N_2 bomb resulted in either intact or completely shattered erythrocytes (Fig. 3C), and similar to the freeze–thaw treatment, submicron particles were almost non-existent (Fig. 3D). Finally, ultrasonication disrupted erythrocytes almost completely (Fig. 3E), producing a concentration of submicron particles higher than the application of freeze–thaw cycles or an N_2 bomb (Fig. 3F). Despite using different ultrasonicators, the size distribution profile of the produced NanoE was similar (data not shown). Extending the length of the ultrasonication treatment from 30 s to 45 s decreased the final particle concentration without affecting the size distribution (Fig. 3G and H). Based on the results, an additional washing step was included in the final protocol to remove any remaining intact cells and larger cell fragments, seen especially in freeze–thaw cycle and N_2 bomb disruptions.

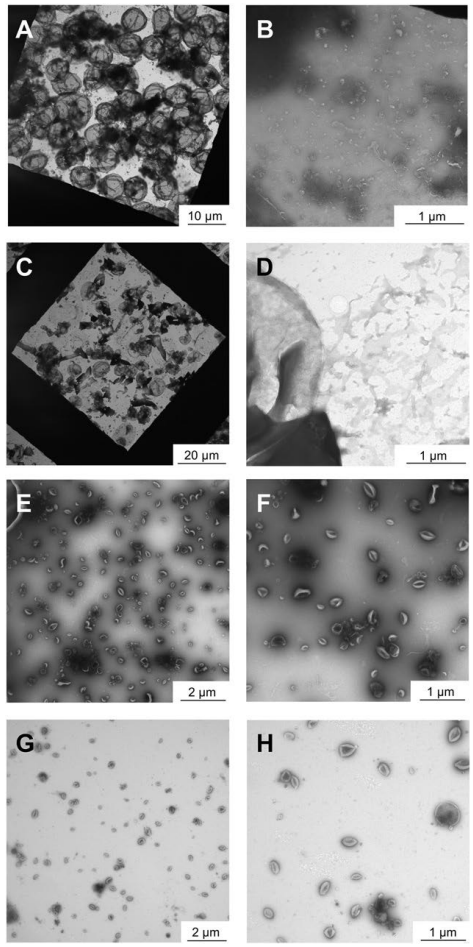

Fig. 3. Representative transmission electron microscope micrographs of erythrocytes disrupted by three consecutive freeze–thaw cycles (A and B), by an N_2 bomb (C and D), by a 30-s ultrasonication (E and F), and by a 45-s ultrasonication (G and H).

3.4. EryEVs vs. NanoE

To examine the usability of NanoE as RM, their physical and biochemical properties were compared with the naturally occurring eryEVs from the same concentrate. The morphology of eryEVs and NanoE was similar as inspected by TEM (Fig. 4A and B). Also, the RI distribution and mean RI (1.37) measured by NTA were similar for eryEVs and NanoE (Fig. 4C). The protein content of NanoE was considerably different from eryEVs as shown by SDS-PAGE (Fig. 4D). Enriched proteins in Western blotting of NanoE vs. eryEVs were, e.g. hemoglobin and Band 3 (data not shown). The size of NanoE was slightly greater than that of eryEVs: 66% of the NanoE population was larger than 200 nm, and the main population (58%) was between 200 and 400 nm, whereas only <30% of eryEVs were larger than 200 nm when determined by NTA (Fig. 4E). The difference in size distribution was also observed in flow cytometry, where a majority of NanoE were found in the same area as 180 nm silica beads in contrast to the smaller eryEVs (Fig. 4F).

Next, flow cytometry was used to determine the fraction of Di-8-ANEPPS positivity and CD235a antigen density of eryEVs and NanoE. Lipid labeling of NanoE by Di-8-ANEPPS showed a higher percentage of labeled particles compared to the eryEVs (67% vs. 45% respectively, $p < 0.05$, Fig. 5). Similarly, the CD235a labeling was significantly higher for NanoE than for eryEVs (58% vs. 21%, respectively, $p < 0.0001$, Fig. 6, fluorescence intensity 68.3 ± 21.6 vs. 40.5 ± 13.0).

3.5. Application of NanoE as a Biological RM to Standardize EV Measurements

To demonstrate the relevance of using a biological RM for the standardization of EV measurements, NanoE was analyzed using three different NTA instruments. The same batch of NanoE was measured using the same settings optimized for the LM14C instrument, which were then applied during measurements with NTA models NS300 and NS500. The size distribution of the detected particles was similar

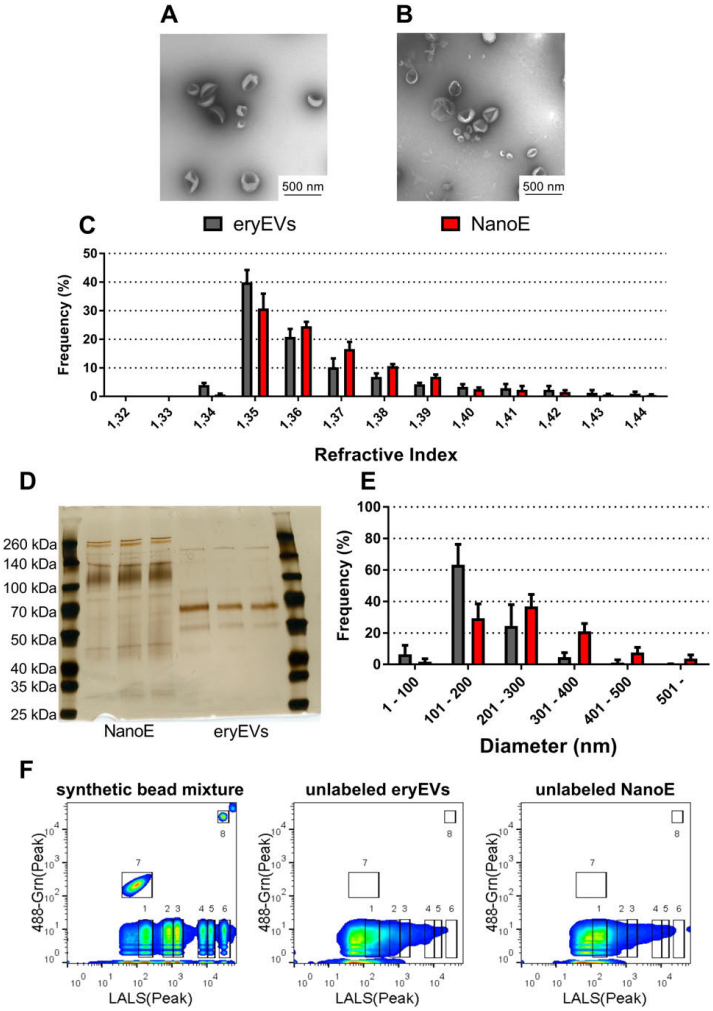

Fig. 4. Comparison of erythrocyte-derived EV (eryEVs) and nanoerythrosomes (NanoE) showing transmission electron microscopy micrographs from (A) eryEVs and (B) NanoE; (C) refractive index measurements; (D) silver-stained SDS-PAGE gel of NanoE and eryEV samples; (E) size distribution measured by nanoparticle tracking analysis; and (F) polystyrene and silica bead mixture, unlabeled eryEVs, and unlabeled NanoE as detected with flow cytometer. Gate 1 = 180 nm silica beads; gate 2 = 240 nm silica beads; gate 3 = 300 nm silica beads; gate 4 = 590 nm silica beads; gate 5 = 800 nm silica beads; gate 6 = 1300 nm silica beads; gate 7 = (fluorescent) 110 nm polystyrene beads; gate 8 = (fluorescent) 500 nm polystyrene beads. Values represent mean ± S.D., $n = 3$ (C) or 18–20 (E).

among the used NTA instruments, with the majority of particles (~60%) ranging from 200 to 400 nm in diameter (Fig. 7A). However, although the same NanoE concentrations were expected to be measured, the obtained NanoE concentrations varied ~40-fold among the different instruments (Fig. 7B).

4. Discussion and Conclusions

The development of RM for EVs is tightly intertwined with the development of the EV analysis methods as both feed each other's advancement. Although progress has been made with different sizes of monodisperse nanoparticles (van der Pol et al., 2014b; Wang et al., 2008), the analyses of complex mixtures of polydisperse particles are challenging (van der Pol et al., 2016). The European Metrology Research Program has initiated important standardization work in anticipation of the rapid momentum of the EV research field and therefore funded METVES, a program focused on metrological characterization of EVs from body fluids. In collaboration with METVES, this study was performed by a national EV research platform funded as an initiative of a Finnish industry- and university-driven research program SalWe-GID, through which multiple end-users are interested in the EV standardization for their improved utilization. The progress in METVES on synthetic RM and the applicable methods for their analysis encouraged us to try to foresee the further needs for biological EV-resembling RM. In order to

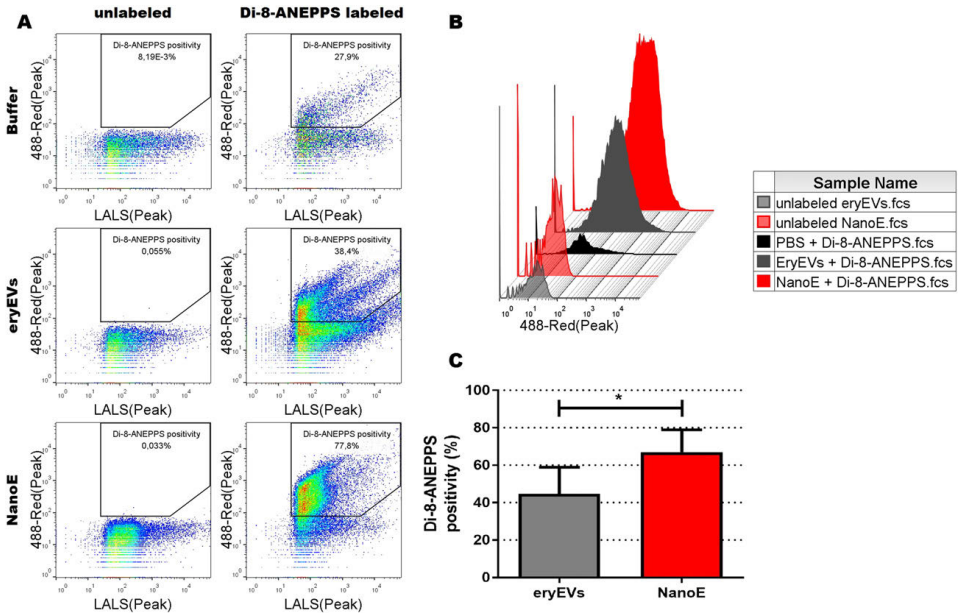

Fig. 5. (A) Apogee A50 dot plots showing the Di-8-ANEPPS labeling of erythrocyte-derived EVs (eryEVs) and nanoerythrosomes (NanoE) compared to negative controls; (B) datagram showing the Di-8-ANEPPS labeling of eryEVs and NanoE compared to negative control; and (C) comparison of Di-8-ANEPPS labeling of eryEVs and NanoE. Values represent mean ± S.D., $n = 6$, $^{*} = p < 0.05$.

gain information on these, a survey was launched. Based on the survey, flow cytometry and synthetic RM were indicated as the most used combination in EV studies, which underpins the problems of RM development. Especially with the flow cytometers, the use of synthetic RM leads to the selection of particles within the size range of cells (van der Pol et al., 2012; Chandler et al., 2011), thereby affecting the conclusions of EV studies particularly from clinical samples such as plasma. Biochemical resemblance to EVs and stability were the most crucial properties for a biological RM according to the laboratories who participated in the survey. As more unusual sources, e.g. plant viruses or marine bacteria, were also acceptable as RM, a wide range of possibilities worth investigating were listed from the literature, if they matched the basic criteria set for RM.

Based on previous results (Anon., n.d.-a), the survey, and the literature review, NanoE was chosen for further development and evaluation as a candidate for biological RM. The optimized method of NanoE production required ultrasonication to disrupt erythrocytes, a method previously used to produce submicron particles from lipids (Lapinski et al., 2007). Upon resealing, the produced particles resembled eryEVs regarding their morphology and RI. The larger size and the enhanced CD235a/Di-8-ANEPPS labeling of NanoE vs. that of eryEVs may be beneficial for their use as EV-RM. Thus, NanoE represents a reasonable option to EV-dedicated flow cytometers and possibly also to earlier models of flow cytometers, which are able to detect 300–700 nm single particles with EV-like RI (van der Pol et al., 2012). The enhanced CD235a positivity may be explained by the instantaneous disruption of erythrocytes, where no selection of surface proteins occurs compared to natural EV budding (Dragovic et al., 2013). This was also supported by the differences of the total protein composition of NanoE and EryEVs. A drawback of NanoE may thus be that since they do not expose common EV surface markers such as CD9, CD63, or CD81 (Yoshioka et al., 2013; Andreu and Yanez-Mo, 2014) nor significant amounts of genomic material, they will not be applicable as RM for methods using these properties as the basis of the analysis. However, the lack of typical EV markers and expression of erythrocyte-specific marker CD235a would enable spiking of EV samples with NanoE, which might be useful in the quantification of EVs from different sources. As such, NanoE can already be utilized as an RM for NTA, TRPS, and flow cytometry.

Comparing the properties of NanoE with those indicated by the survey, NanoE have a matching RI and contain phospholipid membrane and proteins; criteria which fulfil the needs of the majority of the participating EV laboratories. Furthermore, NanoE are relatively stable, safe, and cheap to produce in large quantities from surplus clinical grade erythrocyte concentrates. Although the NanoE population cannot be described as monodisperse, out of the desired properties, monodispersity was ranked as the least important. Monodispersity could be substantially improved by additional preparative steps, thereby allowing the isolation of populations with a narrow size range using different filtration (Zinsser and Tang, 1927), chromatographic (Boing et al., 2014), microfluidics (Ashcroft et al., 2012; Lee et al., 2015), or field-flow fractionation (Petersen et al., 2014; Agarwal et al., 2015) methods. Provided that a monodisperse biological RM could be produced in the future, further characterization by methods such as small-angle X-ray scattering could be, at least hypothetically, used to generate “traceable measurements”, i.e. measurements that could ultimately be related to the SI unit (in this case “metre”) through an unbroken chain of comparisons with known uncertainties (Varga et al., 2014). More realistically, the following step in the development of similarly equivalent standards with NanoE would be the mechanical disruption of platelets or cells from immortalized cell cultures to produce biological RM that would better resemble multiple EV properties, including EV surface markers and internal cargo, and could then be utilized by multiple analytical approaches.

To demonstrate the relevance of biological RM for the standardization of EV measurements, we measured the same batch of NanoE using the same settings on different NTA instruments. The size

distribution of the detected particles was similar, but the obtained NanoE concentrations varied ~40-fold among the NTA instruments, which emphasizes the importance of using an EV-RM. The particle concentration measured by NTA is assumed to be proportional to the mean number of scattering particles in the field-of-view of the microscope, which depends on the intensity and wavelength of the illumination, collection angles of the objective, the sensitivity of the camera with the applied settings (Maas et al., 2015; Gardiner et al., 2013), the analysis software, and the brightness of the scattering particles, which in turn depends on the particle size, refractive index, and concentration (due to multiple and dependent scattering). The differences in the particle concentrations obtained with similar NTA instruments are caused by an inappropriate calibration factor between the mean number of scattering particles in the field-of-view and the provided concentration

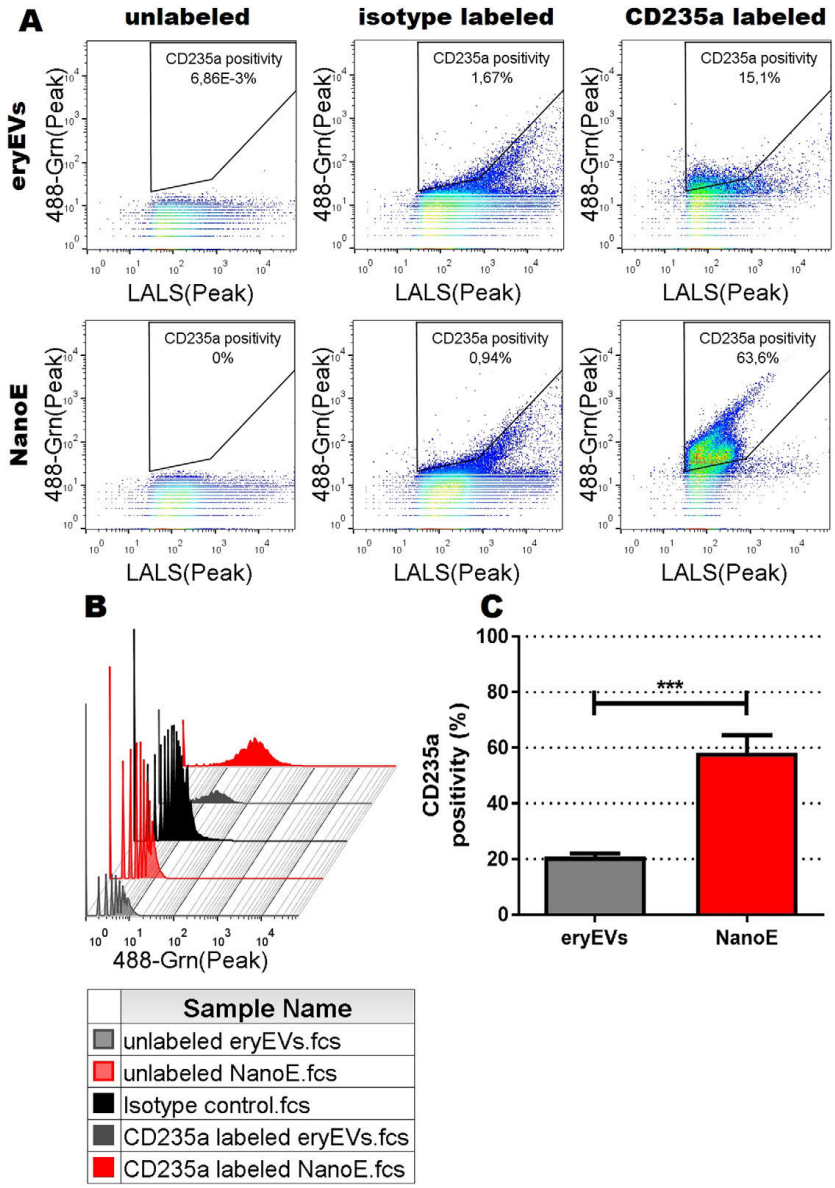

Fig. 6. (A) Apogee A50 dot plots showing CD235a-labeling of erythrocyte-derived EVs (eryEVs) and nanoerythrosomes (NanoE); (B) a datagram showing CD235a-labeling of eryEVs and NanoE; and (C) comparison of CD235a-labeling of eryEVs and NanoE. Values represent mean ± S.D., $n = 6$, *** = $p < 0.0001$.

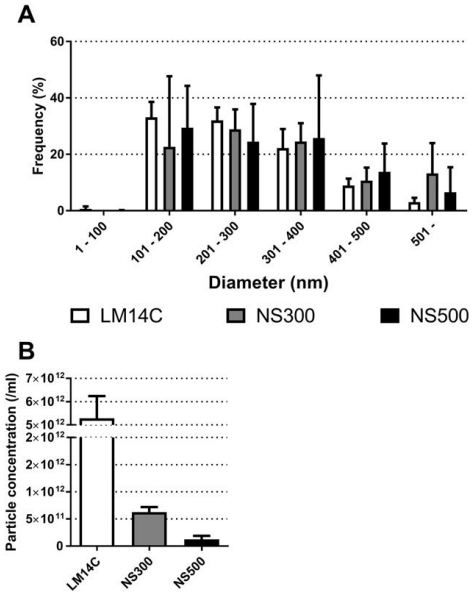

Fig. 7. Characterization of the applicability of the nanoerythrosome standard by measuring the same standard with Nanoparticle Tracking Analysis models LM14C, NS300, and NS500 by (A) particle concentrations and (B) size distribution. Values represent mean ± S.D., $n = 5$.

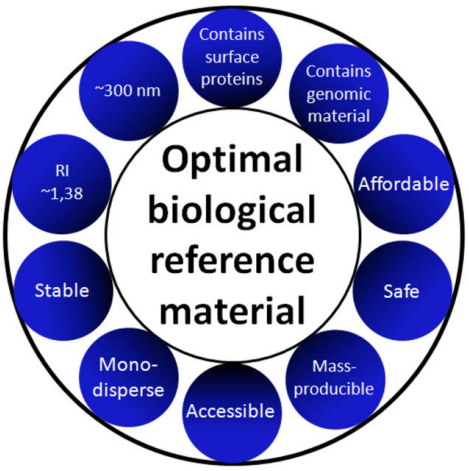

Fig. 8. Optimal properties for a biological reference material for EV studies.

and a lack of knowledge on the diameter of the smallest detectable EVs. Once the concentration of the NanoE can be measured in a traceable way, NanoE can be used to calibrate NTA instruments, i.e. relating the mean number of scattering particles in the field-of-view of NTA to the traceably measured concentration, and defining the smallest detectable EV diameter. In analogy to NTA, other instruments can be calibrated with NanoE to improve the measurement quality within each laboratory. Despite the challenges, EV quantification by particle enumeration is, in most cases, a more accurate way of comparing samples than any indirect EV quantification method such as determination of protein content. The protein content of an EV sample may be independent of the particle number and can vary with the cell activation (Aatonen et al., 2014), the used cell line (Lazaro-Ibanez et al., 2014), and the method by which the protein content is measured (Okutucu et al., 2007). Therefore, direct particle measurements should be favoured in the case of EV quantification and the expressed concentration should be coupled with the knowledge of the detection limits of the instruments/method.

A summary of the optimal properties of a biological RM is presented in Fig. 8, based on the collected information gathered during this study. However, although desirable, it is unlikely that one biological RM would be applicable, not to mention optimal, for all different measurement techniques due to the vast variation in the detection methods (Table 1). Therefore, the search for an optimal biological RM should be approached from a technical perspective, research focus, and considering the EV material. Still, the development of such materials will not be easy.

As the research on EVs progresses, and the use of EVs is pursued in clinical assays and for theranostics, it is crucial to develop various RM to enable precise and reproducible measurements. This will most likely be first achieved by the use of synthetic RM in flow cytometry and in the techniques for EV enumeration. However, the simultaneous development of biological RM would clearly provide additional benefits to the field. Where synthetic RM could be useful for instrument calibration, biological RM could be used for validation of EV measurements. By spreading the RM for common use, the repeatability of studies and the reliability of data will be increased, which in turn will increase the transparency of EV research and improve standardization. Since the discovery of new biological RM for EV studies is a laborious task, it requires the united work of all laboratories and openness. Research networks such as the MEHAD (Extracellular Vesicles in Health and Disease, COST Action BM 1202) and metrological initiatives such as those conducted by METVES, are in a crucial position to take this endeavor to the next level, but ultimately, it is the interest and responsibility of all EV researchers to make this possible.

Abbreviations

eryEVs	erythrocyte-derived extracellular vesicle
EVs	extracellular vesicle
NTA	nanoparticle tracking analysis
NanoE	nanoerythrosome
PBS	phosphate-buffered saline
RI	refractive index
RM	reference material
TEM	transmission electron microscopy
TRPS	tunable resistive pulse sensing

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Appendix A. Supplementary data

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Assessment of Time-Dependent Platelet Activation Using Extracellular Vesicles, CD62P Exposure, and Soluble Glycoprotein V Content of Platelet Concentrates with Two Different Platelet Additive Solutions

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Keywords
Platelet concentrate · Extracellular vesicle · Platelet activation · Platelet additive solution · CD62P · Soluble glycoprotein V

Abstract
Novel analytical measures are needed to accurately monitor the properties of platelet concentrates (PCs). Since activated platelets produce platelet-derived extracellular vesicles (EVs), analyzing EVs of PCs may provide additional information about the condition of platelets. The prospect of using EVs as an auxiliary measure of platelet activation state was investigated by examining the effect of platelet additive solutions (PASs) on EV formation and platelet activation during PC storage. The time-dependent activation of platelets in PCs with PAS-B or with the further developed PAS-E was compared by measuring the exposure of CD62P by flow cytometry and the content of soluble glycoprotein V (sGPV) of PCs by an immunoassay. Changes in the concentration and size distribution of EVs were determined using nanoparticle tracking analysis. A time-dependent increase in platelet activation in PCs was demonstrated by increased CD62P exposure, sGPV content, and EV concentration. Using these strongly correlating parameters, PAS-B platelets were shown to be more activated compared to PAS-E platelets. Since the EV concentration correlated well with the established platelet activation markers CD62P and sGPV, it could potentially be used as a complementary parameter for platelet activation for PCs. More detailed characterization of the resulting EVs could help to understand how the PC components contribute the functional effects of transfused PCs.

Introduction

Platelet concentrates (PC) are manufactured for patients with hemostatic problems, e.g., excessive bleeding or thrombocytopenia in cancer. The average life span of a platelet in blood circulation is 10 days [1], and the storage time of PC is typically 5–7 days [2] mainly due to an increased risk of bacterial contamination during extended storage at room temperature (RT) [3]. Efforts are made to increase the storage time of PCs by developing platelet additive solutions (PASs), improving protocols in PC preparation, pathogen inactivation, and by more detailed quality control [4–6].

A crucial aspect of the quality control of PCs is the determination of platelet activation state. One widely used

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marker of platelet activation is P-selectin (CD62P) exposure of platelets. CD62P is transferred to the platelet plasma membrane through the fusion of α-granules upon activation [7]. Another platelet-specific marker is the soluble form of glycoprotein V (sGPV), which is released from activated platelets through proteolytic cleavage [8]. The transmembrane form of glycoprotein V is located on the platelet surface as part of a complex with glycoproteins Ib and IX, which is the major receptor for von Willebrand factor [9], and it also participates in thrombin [10] and collagen [11] binding. Other current quality control assays of PCs include the quantification of platelet metabolites (glucose, lactate, pH) and dissolved gases (pO_2, pCO_2) as well as the determination of platelet function using the extent of shape change and hypotonic shock response. Also, different platelet parameters, such as the mean platelet volume and platelet count, are commonly monitored to examine the quality of platelets [6].

Besides the platelets' main role in regulation of hemostasis, they have been shown to influence immune responses [7], which is an aspect to consider when PCs are administered to patients. During storage of PCs, platelets liberate a large variety of bioactive components that have been proposed to relate to adverse proinflammatory effects observed in storage lesion [12, 13]. Although several different markers for measuring storage lesion have been suggested, a gold standard to evaluate the usability of PCs for transfusion has not yet been established [6]. Therefore, novel markers are needed to assess the condition of platelets in more detail.

Besides platelets, the PCs contains extracellular vesicles (EVs), which in their majority are derived from platelets, but which also originate from red blood cells and leukocytes residually present in the plasma fraction of PCs [14]. One of the first functions in which platelet-derived EVs were shown to participate was hemostasis [15, 16], implemented by the interaction of coagulation factors on the phosphatidylserine surface of the EVs [17]. Additionally, EVs are considered to be biomarkers of thrombotic and inflammatory diseases as well as cancer [18, 19], and in general EVs have already been shown to mediate several (patho)physiological processes [20, 21]. Furthermore, EVs have been suggested to contribute to adverse transfusion-related reactions [22], underscoring the need to understand the possible effector functions of EVs in transfusion. In addition to the possible effects of the transfused EVs in patients, EV generation during the storage of PCs could be considered as auxiliary parameter to monitor the activation state of platelets, since the generation of platelet-derived EVs is dependent on the aging and activation status of platelets [23, 24].

In the current study, EVs of PC were quantified to see whether the EV content could be utilized as a parameter of the activation state of platelets in PCs together with the recognized platelet activation markers, CD62P exposure of platelets, and sGPV content. This was investigated by examining the platelet activation state in aging PCs with different PASs.

Materials and Methods

Sample Collection

Standard leukocyte-reduced PCs, each derived from buffy coats of four ABO RhD-matched whole blood donations with PAS-B or PAS-E, were obtained from the Finnish Red Cross Blood Service (Helsinki, Finland) and handled anonymously, as accepted by the Finnish Supervisory Authority for Welfare and Health (Valvira, Helsinki, Finland). The exact compositions of PAS-B and PAS-E, also known as PAS-2 and modified PAS-3 [25], respectively, have been reported elsewhere [26].

Sterile sampling was done using 50-mL syringes (Henke-Sass; Wolf GmbH, Tuttlingen, Germany) and 18-gauge needles (Terumo, Tokyo, Japan). Before sampling, the contents of the storage bag's tube were emptied into the storage bag and the PC was mixed by gently turning it from side to side five times. This procedure was repeated three times to obtain a representative sample. After extracting 20 mL of sample via the storage bag's tube, the tube was resealed. The sampling days (d) were d1, d2, d5, and d8 counting from the blood donation (d0), d1 being the production day of PC. The d1 sampling was performed within 2 h once the PCs were available from the production line, approximately at 3 p.m., whereas the d2–d8 samplings were performed at 9 a.m. PCs were stored at 22 °C under constant horizontal agitation.

Determination of CD62P Exposure of Platelets

The CD62P expression on the platelet surface was determined by flow cytometry using 1 mL of PC sample. PC samples were diluted 1:100 (to approximately 1×10^7/mL) using a diluent consisting of the same PAS used for PC production (either PAS-B or PAS-E; SSP or SSP+ [Macopharma, Tourcoing, France]) with 0.5% w/v bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). Fifty microliters of diluted PC sample were labeled using 2 μL of fluorescein isothiocyanate (FITC)-coupled anti-CD41 (FITC mouse anti-human CD41, clone HIP8 [Becton Dickinson, Franklin Lakes, NJ, USA]) and 5 μL of phycoerythrin-cyanine 5 (PE-Cy5)-coupled anti-CD62P (PE-Cy5 mouse anti-human CD62P, clone AK-4 [Becton Dickinson]). For each sample an isotype control sample (50 μL of diluted PC sample labeled with 5 μL of PE-Cy5 mouse IgG1 κ isotype control, clone MOPC-21 [Becton Dickinson]) and a thrombin-activated positive control sample with maximum CD62P expression (50 μL of diluted PC sample labeled with 2 μL of CD41-FITC and 5 μL of CD62P-PE-Cy5 and activated with 1 IU/mL thrombin [Roche, Basel, Switzerland]) were prepared. Samples and controls were labeled and analyzed in BD TruCount tubes (Becton Dickinson) containing a known number of fluorescent beads. Samples were analyzed in singlicates since previously the CD62P measurements had been found well repeatable [27]. After labeling samples were mixed, incubated for 20 min at RT in the dark, further diluted with 500 μL of diluent, and stored in the dark until analysis.

The samples and controls were analyzed with a Navios flow cytometer (Beckman Coulter, Brea, CA, USA) at "high-flow" speed. The forward (FS) and side scatter detectors' (SS) volt and gain settings had been adjusted such that the platelet population was centered in the FS-SS dot plot (at around 10^1) and the fluorescence detectors' FL1 (FITC) and FL4 (PE-Cy5) settings such that the detected fluorescence signals were well within the displayed

ranges for all samples. The TruCount beads were gated based on their fluorescence in FL1, FL2, and FL3 channels and a platelet gate had been defined in the FL1-SS dot plot. For each sample 5,000 bead events were acquired, corresponding to about 60,000 platelet events. The gated platelet population was used to calculate the percentage of CD62P-positive platelets, defined as:

- Based on the isotype control, a threshold was set to include 1% of all events with the highest fluorescence in the FL4 channel. All events with FL4 fluorescence above this threshold were defined as CD62P-positive in comparison to the isotype control.
- Based on the positive control, a threshold was defined to include 95% of the thrombin-activated platelet population with the highest fluorescence. All platelets with FL4 fluorescence above this threshold were considered CD62P-positive in comparison to the positive control.

Platelet activation state was expressed in relation to both isotype and positive control as percentage of gated platelet population above the respective CD62P positivity threshold.

Quantification of sGPV

The quantification of sGPV was performed as reported previously [6]. Briefly, 1 mL of the PC was centrifuged (Biofuge 13; Heraeus Sepatech, Hanau, Germany), first at 3,600 *g* at RT for 15 min and the supernatant again at 11,000 *g* at RT for 5 min (Biofuge 13). The supernatant was transferred to new tubes in 500-µL aliquots and stored at –70 °C until sGPV quantification with a commercial kit (Asserachrom; Diagnostica Stago, Asnières sur Seine, France). For the measurement, samples were diluted 1:80–1:640 using phosphate buffer provided with the kit, and the amount of sGPV was expressed as pmol/10^9 platelets.

Isolation of EVs

A total of 17 mL of PC was used for EV isolation. To prevent platelet activation, Anticoagulant Citrate Dextrose Solution pH Eur Solution A (Terumo BCT, Lakewood, CO, USA) and Apyrase (Sigma-Aldrich) were added to the final concentrations of 4.25% v/v and 2 U/mL, respectively, and the PC was diluted 1:4 with phosphate-buffered saline (PBS) (Thermo Fisher, Waltham, MA, USA). The diluted PC was centrifuged at 650 *g* at RT for 7 min (Eppendorf centrifuge 5810R; Eppendorf, Hamburg, Germany) without brake, and the supernatant was centrifuged at 1,560 *g* at RT for 20 min (Eppendorf centrifuge 5810R). The residual platelet content of the supernatant was reduced to 1×10^6 platelets/mL, as confirmed with Coulter Cell Counter T-540 (Beckman Coulter). To extract the whole EV population from the PC, the supernatant was ultracentrifuged at 100,000 *g* at 4 °C for 1 h (MLA-50 rotor, k-factor 92; Beckman Coulter). The supernatant was carefully decanted and remaining supernatant was removed with a pipette, after which the EV sample was resuspended into 200 µL of PBS and stored in Protein LoBind tubes (Eppendorf) at –70 °C until analysis.

Quantification and Size Determination of Particles in EV Samples

The concentration and size distribution of particles in EV samples was determined using nanoparticle tracking analysis. The LM14C model used was equipped with a 70-mW violet (405 nm) laser (Malvern Instruments Ltd., Malvern, UK) and a sCMOS camera (Hamamatsu Photonics K.K., Hamamatsu, Japan). Data were captured using camera level 14, and three videos of 90 s were recorded, manually mixing the sample between measurements. EV samples from PAS-B PCs were diluted 1:1,000, 1:2,000, 1:5,000, and 1:10,000 with filtered (0.2 µm) PBS on d1, d2, d5, and d8 samples, respectively, and EV samples from PAS-E PCs were diluted 1:1,000, 1:2,000, 1:5,000, and 1:5,000–1:10,000 on d1, d2, d5, and d8 samples, respectively. Data analysis was performed with threshold 5 and gain 10. Data were recorded and analyzed with NanoSight software version 3.0 (Malvern Instruments Ltd.). The data were reported as EV concentration of the PC on the sampling day by calculating the particle content of the EV sample using the determined particle concentration and taking into account that the particles were isolated from a 17-mL sample, considered as a representative sample of PC.

Staining and Characterization of EV Samples on ImageStream®X Mark II

EV samples from PAS-E were labeled with Alexa Fluor 488 C_5 maleimide (Invitrogen, Carlsbad, CA, USA) for 60 min at RT as described previously [28]. Excess maleimide was removed by using exosome resin spin columns (Invitrogen) which were prepared according to the manufacturer's instructions. Maleimide labeling without EVs was performed in a parallel fashion to confirm the dye retention by columns and to get "mock" controls for the experiments.

Different fluorescent stains for further characterization of EVs were used according to the manufacturer's instructions. Antibodies were: CD41a AF647 (clone HIP8; BioLegend, San Diego, CA, USA), CD45 PerCP-Cy5.5 (clone 2DI; BioLegend), CD63 BV510 (clone H5C6; BD BioScience, San Jose, CA, USA), and CD235a Pacific Blue (clone HI264; BioLegend). Apolipoprotein A contamination was surveyed with ApoA1 PerCP (BioSite, Täby, Sweden). Antibodies were incubated for 30 min at RT in the dark in PBS.

Maleimide 488 and fluorescent-positive EVs were detected using a 12-channel Amnis® ImageStream®X Mark II (EMD Millipore, Burlington, MA, USA) imaging flow cytometer. Samples were acquired at 60× magnification with low flow rate/high sensitivity. The integrated software INSPIRE® (EMD Millipore) was used for data collection. The instrument and INSPIRE software were set up as follows: excitation lasers 488, 642, and 785 and channel 01 (Ch01) and Ch09 (bright field), Ch06 (scattering channel), plus fluorescence channels Ch02, Ch05, Ch07, Ch08, and Ch011 were activated for signal detections.

At least 10,000 events for each sample were acquired. Positive events for maleimide 488 were gated based on the intensity values and used for further analysis. Single-color controls were used for compensation and unlabeled EVs were used to determine the autofluorescence. Buffers with and without antibody/maleimide 488 molecules were used to determine the background noise. Compensated data files were analyzed using image-based algorithms available in the IDEAS® statistical analysis software package (version 6.2.188.0).

Statistical Analysis

Kruskal-Wallis test together with Dunn's multiple comparison test to take into account the effect of multiple testing was used to determine the significance of the results within PASs, and p values <0.05 were considered significant. To determine the significance between PASs on d5 sample Mann-Whitney test together with Bonferroni correction was used. Spearman correlation coefficient and related p value together with R^2 value of the standard curve was used to determine the correlation between the different platelet activation parameters. All statistical analyses were performed using GraphPad Prism v. 6.07 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

CD62P Exposure of Platelets

A statistically significant, time-dependent increase in CD62P exposure was observed during the 8-day storage

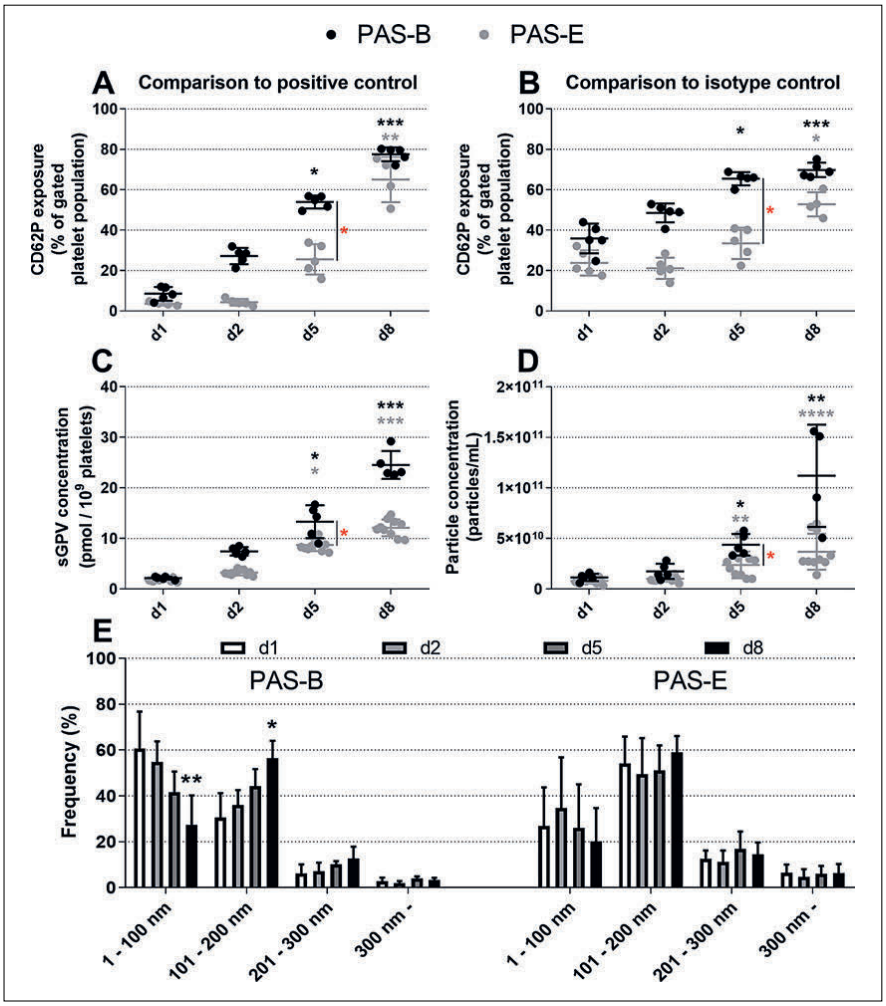

Fig. 1. Quality control markers used for the evaluation of platelet activation during storage of PCs with PAS-B and PAS-E. **A**, **B** Time-dependent changes in the CD62P exposure of platelets when compared to a positive (**A**) or an isotype control (**B**). **C** sGPV production of platelets. **D**, **E** Concentration (**D**) and size distribution (**E**) of particles in the EV samples isolated from PCs. Statistical difference within a given PAS is indicated with black and grey asterisks for PAS-B and PAS-E PCs, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to d1 using Kruskal-Wallis test with Dunn's multiple comparison. Statistical difference between PAS-B and PAS-E PCs on d5 (Bonferroni-adjusted $p < 0.05$ using Mann-Whitney test with Bonferroni correction is indicated with a red asterisk). Bars represent mean with standard deviation in **A–D**; columns present mean and bars standard deviation in **E**. Data were acquired in three independent experimental settings, $n = 4–5$ (PAS-B in all figures, PAS-E in **A** and **B**) or $n = 10$ (PAS-E in **C–E**). d, day; EV, extracellular vesicle; PAS, platelet additive solution; PCs, platelet concentrates; sGPV, soluble glycoprotein V.

period. The exposure of CD62P in the PAS-B stored platelets increased from 8.5 to 78% ($p = 0.0226$ and $p = 0.0002$ on d5 and d8, respectively) when compared to the positive, thrombin-activated control (Fig. 1A). In PAS-E platelets, the average CD62P exposure increased from 3.6 to 71% ($p = 0.0028$ on d8) compared to the positive control (Fig. 1A).

When CD62P exposure was determined by comparison to the isotype control, the CD62P exposure of PAS-B platelets increased from 36 to 70% ($p = 0.0177$ and $p =$

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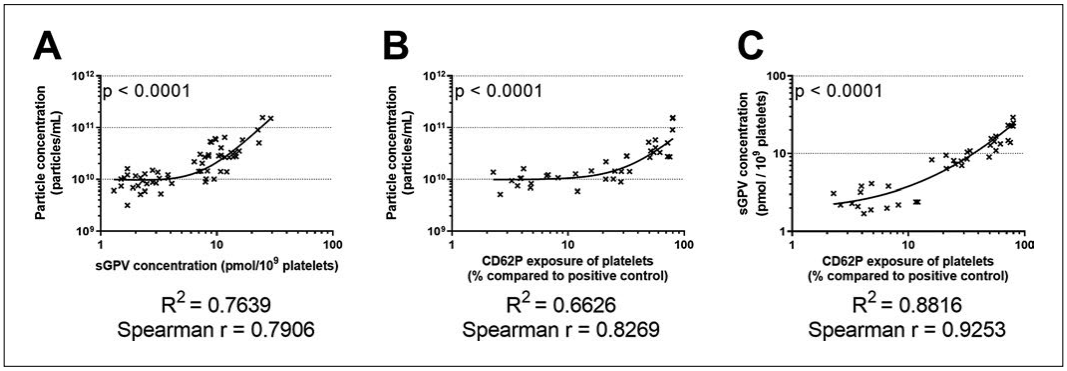

Fig. 2. Correlation analysis of the three different markers for platelet activation. **A** Particle concentration in the EV samples and sGPV production of platelets. **B** Particle concentration in the EV samples and CD62P exposure of platelets when compared to a positive control. **C** sGPV production and CD62P exposure of platelets when compared to a positive control. The figure was compiled using data from both PAS-B and PAS-E PCs acquired from three independent experimental settings. EV, extracellular vesicle; PAS, platelet additive solution; PCs, platelet concentrates; sGPV, soluble glycoprotein V.

0.0005 on d5 and d8, respectively) and from 24 to 53% ($p = 0.0117$ on d8) in the PAS-E platelets (Fig. 1B).

Regardless of the CD62P exposure determination method, on d5, the last day when the PC can be transfused to patients, platelets in PAS-B PCs exposed more CD62P than platelets in PAS-E PCs (Bonferroni-adjusted $p = 0.0016$ for both) (Fig. 1A, B).

sGPV Content in PCs

The sGPV content of PAS-B PCs increased from the average of 2.1 to 24.5 pmol/10^9 platelets during storage ($p = 0.0225$ and $p = 0.0002$ on d5 and d8, respectively) (Fig. 1C). Also in the PAS-E PCs, the increase in sGPV was statistically significant, but more subtle, as the sGPV concentration increased from 1.8 to 12.1 pmol/10^9 platelets ($p = 0.0224$ and $p = 0.0002$ on d5 and d8, respectively) during the 8-day storage (Fig. 1C). The sGPV content of PCs was significantly higher in PAS-B PCs than PAS-E PCs on d5 (Bonferroni-adjusted $p = 0.0158$) (Fig. 1C).

Concentration and Size Distribution of Particles in EV Samples

The particle concentration in EV samples of PAS-B PCs significantly increased during the 8-day storage period from 1.1×10^{10} particles/mL on d1 to 1.3×10^{11} particles/mL on d8 ($p = 0.0292$ and $p = 0.0021$ on d5 and d8, respectively) and in the PAS-E PCs from 7.9×10^9 particles/mL on d1 to 3.7×10^{10} particles/mL on d8 ($p = 0.0019$ and $p < 0.0001$ on d5 and d8, respectively) (Fig. 1D). Both the PAS-B and PAS-E PCs initially had similar particle concentration in EV samples, but from d2 onwards the particle concentration in EV samples of PAS-B PCs was higher compared to PAS-E PCs (Bonferroni-adjusted $p = 0.016$ on d5) (Fig. 1D).

Table 1. Frequency of surface markers identified from maleimide-positive particles of extracellular vesicle samples isolated from platelet concentrate on days 1 and 8

	Day 1	Day 8
ApoA1	0.8%	1.4%
CD41	68.7%	88.0%
CD45	0.6%	2.6%
CD63	29.0%	57.7%
CD235a	5.1%	2.1%

The size distribution of particles in EV samples changed significantly during aging only in the PAS-B PCs (Fig. 1E). Initially, 61% of the particles were <100 nm in diameter, but after 8 days of storage, the percentage of particles <100 nm had decreased to 27% ($p = 0.0070$). Consequently, the percentage of particles with a diameter of 101–200 nm was initially 31%, which increased to 56% on d8 ($p = 0.0484$). In contrast to PAS-B, no significant alteration in the size distribution of particles in EV samples was observed in PAS-E PCs (Fig. 1E).

Characteristics of EVs Isolated from PCs

From all the maleimide-positive particles in the EV samples, the majority expressed CD41, indicating that the EVs isolated from the PCs are mainly derived from platelets. Also, the EV marker CD63 was abundantly present, and the number of CD41- and CD63-positive particles

increased during the 7-day storage. Besides the platelet-derived particles, minute amounts of leukocyte- and erythrocyte-derived particles and ApoA1 were detectable in the EV samples (Table 1).

Correlation of EV Sample Particle Concentration with CD62P Exposure and sGPV Content

A strong positive correlation was observed between the particle concentration of EV samples and the sGPV content of PCs (R^2 = 0.7639, Spearman r = 0.7906 with p < 0.0001) (Fig. 2A), and a very strong positive correlation was observed between the particle concentration of EV samples and the CD62P exposure of platelets (R^2 = 0.6626, Spearman r = 0.8269 with p < 0.0001) (Fig. 2B) and between the sGPV content of PCs and the CD62P exposure of platelets (R^2 = 0.8816, Spearman r = 0.9253 with p < 0.0001) (Fig. 2C).

Discussion

For a few decades, PCs have been prepared with PAS together with some plasma. Initially only plasma-containing PCs were favored due to better functionality (estimated by corrected count increments and bleeding) compared to only PAS-containing PCs [29]. The disadvantages of plasma-containing PCs include increased incidence of adverse transfusion-related reactions, mainly allergic reactions, but possibly also transfusion-related acute lung injury and ABO-mismatched hemolysis [26]. Currently, approximately 30% of the volume of PAS-containing PCs still contains plasma to maintain platelet functionality [30], but the development of PASs has resulted in a notable improvement of platelet quality and functionality, leading to experimentations with decreased content of plasma in PCs with PASs [31]. At the moment, PAS-E is considered to be the best PAS developed, having similar platelet functionality to the PCs with plasma in terms of corrected count increment [29], and it has even been hypothesized whether with the addition of further components such as glucose [30], the advantage of plasma could be surpassed in favor of the PAS-only PCs. The driving force behind reducing the plasma content in PCs is the potential decline in the incidence of adverse transfusion-related reactions. Additionally, the leftover plasma could be utilized for fractionation to produce other transfusable products [26].

All common PASs contain NaCl and acetate [26]. NaCl is added in varying amounts to adjust PC osmolarity, and acetate is added for two reasons: firstly, to provide an alternative energy source in addition to glucose, as it reduces lactate production and consequently influences the pH of the PC; secondly, during the enzymatic processing of acetate, carbon dioxide is formed, which further reacts with water to form bicarbonate providing increased buffer capacity to PCs [32]. Most PASs also contain citrate as an anticoagulant, which provides yet another energy source and added buffer capacity [26, 33]. Furthermore, PAS-E contains phosphate, potassium, and magnesium, whereas PAS-B does not [26]. Phosphate is added to PCs for improved buffer capacity and to stimulate platelet glycolysis [34]. PASs with different compositions have been extensively tested, and addition of potassium and magnesium has been connected to decreased cytokine [35] and lactate [36, 37] production as well as decreased CD62P [37, 38] and phosphatidylserine [38] exposure of platelets. Our results on time-dependent platelet activation are in line with these previous findings as based on the three assessed platelet activation markers (CD62P exposure of platelets together with sGPV and EV content of PCs), platelets in PAS-B PCs were more activated than PAS-E PC platelets on d5, the last day when PC could be transfused to patients. A significant increase in the activation state of the PAS-B platelets was detected with all three activation markers at d5 sampling. For PAS-E PCs a significant increase in sGPV content and EV particle concentration was observed on d5 as well, whereas a significant increase in CD62P exposure was observed only on d8. Although it is unclear how altered PAS composition affects platelet activation, the mechanism might involve membrane potassium movement and permeability [39]. Similarly to CD62P and sGPV, a time-dependent increase in EV concentration in PCs was observed, in line with previously published results [40, 41]. Based on the current data, EV concentration correlated well with the sGPV content and the CD62P exposure of platelets, indicating that EV concentration could be used to indicate platelet activation in PCs.

As shown previously, determination of both the CD62P exposure of platelets and the sGPV content in PCs were sensitive and reproducible methods to detect platelet activation [6, 27]. As an additional advantage of these methods, the maximum extent for both parameters can be determined, which helps to estimate the platelet activation state by giving either a relative or an absolute [42] boundary value (for CD62P and sGPV, respectively). Contrary to CD62P and sGPV measurements, it is not possible to generate an accurate control for a maximum EV production as different agonists produce varying amounts of platelet-derived EVs [23]. Although current PC manufacturing processes ensure minimal cell contamination, EVs from erythrocytes, platelets, and leukocytes are present in PCs already due to the plasma component of the PCs, as shown in this study and by others [14]. It is difficult to determine whether the platelet-derived EVs are produced due to

aging-related platelet activation, as a result of interaction of buffy coat components during storage, or even due to an apoptosis-like process [43]. The interaction of buffy coat EVs and platelets might explain the platelet activation to some degree [44] and consequently the high variation in the particle concentration of EV samples from PCs especially seen in the d8 samples. Additionally, considering the variance in donors [45, 46], current EV sample preparation methods [47], and the lack of standardized and accurate EV quantification methods [48–50], it must be stressed that although EV concentration seems to be a potential marker of platelet activation, significant development and standardization will be needed before the current methods can be replaced to determine platelet activation state in PCs. The authors would like to underline that EVs could still be used as a complementary platelet activation marker to CD62P and sGPV.

In addition to EV concentration being a marker for platelet activation similarly to CD62P or sGPV, EVs could also provide qualitative information of PC aging and possibly even functionality. Besides influencing the size [23, 51] and the molecular cargo [23, 24, 52] of produced EVs, platelet activating conditions have been shown to affect the subsequent function of produced EVs [53], and future studies could concentrate on the qualitative information provided by PC-derived EVs. In the current study, we observed a time-dependent increase in the size distribution of particles in the EV samples from platelets stored in PAS-B. Since platelet activation was influenced by PAS composition, it may also have an influence on the produced EVs. Another possible explanation for the altered size could be an artefactual clumping of EVs, which has been shown previously [54]. However, the effect was only subtle compared to the size of for instance EV doublets and without a corresponding decline in the particle concentration, so formation of stickier EVs is unlikely to explain the current results. A change in the EV population in the PC, reflected by size change, could also have functional effects upon transfusion [55]. To understand the potential effects of EVs in storage lesion, adverse transfusion-related reactions, or immunomodulatory functions in general, it will be necessary to carefully examine the molecular composition of EV populations by lipidomics, proteomics, or metabolomics [56–58]. Moreover, characterizing the PC-derived EVs could also be a step towards personalized transfusion treatments, where patients could be targeted to receive PCs that would best suit their needs [45, 46], e.g., PCs that have more procoagulant potency in the case of severe bleeding. By doing this, the utilization of PCs could be optimized, leading to less wasted PCs and hopefully to transfusions with less adverse transfusion-related reactions.

In conclusion, EVs may be a useful tool in the quality control of PCs in the future, and the molecular characterization of EVs could provide more information about the state and usability of the PCs, ultimately benefitting patients receiving transfusions.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Lipid mediators in platelet concentrate and extracellular vesicles: Molecular mechanisms from membrane glycerophospholipids to bioactive molecules

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ABSTRACT

Platelets are collected for transfusion to patients with different haematological disorders, and for logistical reasons, platelets are stored as concentrates. Despite carefully controlled conditions, platelets become activated during storage, and platelet concentrates (PlaCs) may cause adverse inflammatory reactions in recipients. The time-dependent changes in the lipidome of clinical PlaCs, platelets isolated from PlaCs, and extracellular vesicles (EVs) thereof were examined by mass spectrometry. The relative amount of arachidonic acid containing glycerophospholipids, especially those in the phosphatidylethanolamine and phosphatidylserine classes during storage, but the relative amount of other polyunsaturated fatty acid containing glycerophospholipids remained stable in all sample types. These changes were not directly translated to lipid mediator (LM) profile since the levels of arachidonic acid-derived proinflammatory LMs were not specifically elevated. Instead, several monohydroxy pathway markers and functionally relevant LMs, both proinflammatory and proresolving, were detected in the PlaCs and the EVs, and some representatives of both kind clearly accumulated during storage. By Western blot, the key enzymes of these pathways were shown to be present in platelets, and in many cases, EVs. Since the EVs were enriched in the fatty acid precursors of LMs in their (phospholipid) membranes, harboured LM-producing enzymes, contained the related monohydroxy pathway markers, and secreted the final LM products, PlaC-derived EVs could participate in the regulation of inflammation and healing, and thereby aid the platelets in exerting their essential physiological functions.

1. Introduction

Besides being structural components and the means for storing energy, fatty acids and membrane lipids play significant roles in cell signalling pathways [1–3]. For platelets, membrane lipids are a prerequisite for optimal functioning and therefore, their membrane lipidome is under constant enzymatic remodelling and regulation [4]. Glycerophospholipid (GPL)-derived polyunsaturated fatty acids (PUFAs) such as arachidonic acid (20:4n-6, AA), eicosapentaenoic acid (20:5n-3, EPA), n-3 docosapentaenoic acid (22:5n-3, DPA), and docosahexaenoic acid (22:6n-3, DHA) are used to produce lipid mediators (LMs), which play vital roles in the different phases of inflammation, including the resolution and healing process [5–8]. In contrast to the well-characterized, time-dependent platelet GPL alterations and related mechanisms [9–12], the role of LMs in platelets is incompletely understood. Specific LMs, such as thromboxane (Tx)A_2, prostaglandin (PG)D_2 and PGE_2, have well known functions in platelets [13–15], but the biological actions of the recently discovered proresolving LMs, such as resolvins (Rvs), protectins and maresins, remain of interest [16–19] in both platelets and their extracellular vesicles

Abbreviations: EV, extracellular vesicles; LM, lipid mediator; NTA, Nanoparticle Tracking Analysis; PlaC, platelet concentrate; SPM, specialized proresolving mediator

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(EVs).

Platelet concentrates (PlaC) are widely used in clinical settings to treat patients with thrombocytopenia (low platelet count), to replace platelets in case of massive bleeding and to prevent bleeding in patients with cancer, malignant blood diseases, or other malfunctions of platelets. The lifespan of a platelet in the circulation is typically 8 to 10 days [20], and currently, the storage time of clinical PlaCs is 5 to 7 days [21]. In addition to replenishing the patient's platelet count to a haemostatically functional level, clinical PlaCs may contribute to the overall immunological status of a patient, as platelets communicate with and modulate other cells involved in immunological reactions [15,22–27].

One way of cell communication is mediated through the secretion of EVs, which are protein-, lipid-, carbohydrate-, nucleic acid-, and metabolite-containing lipid bilayered particles produced by most cells [28,29]. Platelets are sensitive cells that are activated in multiple ways including mechanical stress [30], chemical agonists [31], and ageing [32]. When activated, platelets produce EVs with an activation-dependent composition [33,34]. During storage, platelet activation and the concentration of EVs increase in the PlaC [35–37], which may affect the recipient's transfusion response. The molecular mechanisms underlying adverse transfusion responses, such as allergic and febrile nonhemolytic reactions, are still poorly understood [38]. Analogously, the increasing numbers of EVs in ageing erythrocyte concentrates have been associated with a proinflammatory host response [39].

During ageing, at least two types of phospholipid-related alterations occur in platelets within the PlaC: following the depletion of ATP, membrane phospholipid asymmetry cannot be maintained by lipid translocating enzymes and is eventually lost [40], which is an event tied to EV formation [41]. In addition to the defective control of transbilayer lipid distribution, lipid peroxidation and EV secretion cause losses of phospholipid content [12,42]. As the acyl chains of membrane GPLs may be cleaved and converted to LMs, it is important to investigate the abundance of the molecular species within the different GPL classes along with the enzymes required for LM biosynthesis. Depending on the presence of precursor PUFAs and the enzymes that modify them, the resulting downstream LMs exhibit proinflammatory properties, proresolving properties, or have dual functions [7,43,44]. Depending on these functions, LMs are generally categorized as proinflammatory mediators or specialized proresolving mediators (SPMs). SPMs have been shown to modulate the resolution, i.e., the active dampening phase of inflammation, in several disease models [5,45,46]. It has been demonstrated that only pico to nanomolar concentrations of SPMs are required to exert their effects on cellular functions [47], and the recent technological development has now enabled the discovery of novel SPM structures. As PlaC lipids may have an impact on the immunological cells of a patient receiving the clinical PlaC, it is crucial to comprehend the time-dependent LM alterations in the platelets and EVs of the clinical PlaCs by studying i) how the shifts in the GPL profiles are translated to the LM profile, ii) whether certain LMs or their pathway markers are transported in EVs or platelets, and iii) to what extent the LMs originated from platelets or their EVs influence the surrounding cells. By solving these questions, we can begin to elucidate the lipid-mediated effects of PlaC transfusion from an immunological perspective.

In the current study, time-dependent alterations in GPL composition during the storage of clinical PlaCs were investigated from three sample types: PlaCs as a whole, platelets, and PlaC-derived EV samples. Second, the presence of specific enzymes required for LM production was verified. Furthermore, the LMs of the PlaCs and EVs were profiled to unravel whether certain LMs become more prevalent as a function of time.

2. Materials and methods

2.1. Sample preparation

In total, five standard leukocyte-reduced clinical-grade PlaCs, each derived from four buffy coats of ABO RhD-matched whole blood donations, were obtained from the Finnish Red Cross Blood Service (Helsinki, Finland) and handled anonymously, as accepted by the Finnish Supervisory Authority for Welfare and Health (Valvira, Finland).

Sterile sampling was done using 50 mL syringes (Henke-Sass, Wolf GmbH, Tuttlingen, Germany) and 18-gauge needles (Terumo, Tokyo, Japan). Before sampling, the contents of the storage bag's tube were emptied into the storage bag, and the PlaC was mixed by gently turning it from side to side 5 times. This procedure was repeated 3 times to obtain a representative sample. After extracting 20 mL of sample via the storage bag's tube, the tube was resealed. The sampling days (d) were d1, d2, d5, and d8 counted from blood donation (d0), where d1 was the production day of a PlaC. The d1 sampling was performed within 2 h after the PlaCs were available from the production line, approximately at 3 p.m., but the d2–d8 samplings were performed at 9 a.m. PlaCs were stored at 22 °C under constant horizontal agitation and subjected to standard quality control of the Finnish Red Cross Blood Service (visual inspection of PlaC, and determination of glucose concentration, lactate concentration, potassium concentration, pCO_2, pO_2, pH, and residual leukocyte count). Additionally, microbiological cultures were prepared from the PlaCs after the final sampling day to exclude possible microbial contamination (data not shown).

Aliquots of 250 µL of untreated PlaC were snap frozen and placed under argon gas to store the lipids. The PlaC samples were stored at −70 °C until the analysis. A total of 17 mL of PlaC was used to prepare an EV sample. To prevent platelet activation, Anticoagulant Citrate Dextrose Solution pH Eur Solution A (Terumo BCT, Lakewood, CO, USA) and Apyrase (Sigma-Aldrich, St. Louis, MO, USA) were added at final concentrations of 4.25% v/v and 2 U/mL, respectively, and the PlaCs were diluted 1:4 with phosphate-buffered saline (PBS (Thermo Fisher Scientific, Waltham, MA, USA)). The diluted PlaCs were centrifuged at 650 × *g* at RT for 7 min (Eppendorf centrifuge 5810R, (Eppendorf, Hamburg, Germany)) without braking, and the pelleted platelets were placed under argon and snap frozen. The supernatant was centrifuged at 1560 × *g* at RT for 20 min (Eppendorf centrifuge 5810R), and the platelet content of the supernatant was reduced to 1×10^6 platelets/mL, as confirmed with Coulter Cell counter T-540 (Beckman Coulter). To extract the whole EV population from the PlaC, the supernatant was ultracentrifuged at 100000 × *g* at 4 °C for 1 h (MLA-50 rotor, k-factor 92 (Beckman Coulter)). The supernatant was carefully decanted and the remaining supernatant was removed with a pipette, after which the EV pellet was resuspended into 200 µL of PBS and divided into two aliquots of 90 µL for lipid analysis and a 20 µL aliquot for Nanoparticle Tracking Analysis (NTA) in Protein LoBind tubes (Eppendorf). Immediately after preparing the aliquots, samples were snap frozen, placed under argon and stored at −70 °C until lipid analysis.

2.2. Nanoparticle Tracking Analysis

The particle number and size distribution of the particles in the EV samples were analysed with NTA instrument LM14C equipped with a violet (405 nm, 70 mW) laser (Malvern Instruments Ltd., Malvern, UK) and an sCMOS camera (Hamamatsu photonics K.K., Hamamatsu, Japan) at controlled temperature of 22.0 °C and camera level 14 using Nanosight software 3.0 (Malvern Instruments Ltd.). EV samples were diluted 1:1000, 1:2000, 1:5000 and 1:10000 with filtered (0.2 µm) PBS on d1, d2, d5 and d8 samples, respectively, and three videos of 90 s were recorded from samples, mixing the sample manually between measurements. Data were analysed with NanoSight NTA 3.0 software

using a detection threshold 5 and a gain of 10.

2.3. EV characterization with Amnis imaging flow cytometer

EV samples were carboxyfluorescein succinimidyl ester (CFSE)-labelled using 20 μM CFSE diluted from 5 mM CFSE in DMSO and further in D-PBS. 100 μL of EV samples, 150 μL PBS, and 250 μL 20 μM CFSE were incubated for 15 min in the dark at +37C. Samples were purified using SEC prepared as in [48], and fractions 8 and 9 were collected and used for the analysis. CFSE positive EVs were analysed using excitation laser 488 in 12 channel Amnis® ImageStream®X Mark II (EMD Millipore) imaging flow cytometer. Samples were acquired at 60 × magnification with low flow rate/high sensitivity. The integrated software INSPIRE® (EMD Millipore) was used for data collection. The instrument and INSPIRE software were set up as follows: channels 01 and Ch9 (bright field, BF), Ch06 (scattering channel), plus fluorescence channel Ch02 were activated for signal detection. Single colour controls were used for compensation and unlabeled EVs were used to determine the auto fluorescence. Buffer with and without CFSE molecules were used to determine the background noise. Compensated data files were analysed using image-based algorithms available in the IDEAS® statistical analysis software package (version 6.2.188.0).

2.4. Western blotting

To ensure sufficient sample material for Western blot analysis, a separate sampling was conducted, where the sampling volume from PlaC was increased to 50 mL. Samples were collected from two PlaCs on d1 and d8, and sample collection and processing were conducted similarly as above. One tablet of cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) was dissolved into 1.5 mL of PBS, and a volume equal to 1/6 of the sample volume was added to samples before determining the protein content using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The samples containing 25 μg of protein for each well were prepared together with 4× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 10% 2-mercaptoethanol (Sigma-Aldrich) and boiled for 5 min. Then samples were loaded onto Mini-PROTEAN TGX Stain-Free protein gels with a 4–20% gradient (Bio-Rad) in biological duplicates together with Precision Protein Plus WesternC Blotting Standard (Bio-Rad). Gels were run for 50 min at 170 V in 1× Tris/Glycine/SDS Buffer (Bio-Rad), and proteins were blotted for 20 min with 1.3 A up to 25 V using a semi-dry blotting machine Trans-Blot Turbo (Bio-Rad), 1× Transfer Buffer (Bio-Rad), including 20% methanol (Merck), and Trans-Blot Turbo Mini Nitrocellulose Transfer Packs (Bio-Rad), where the original 0.2 μm nitrocellulose membrane was replaced with a 0.45 μm nitrocellulose membrane (Bio-Rad). Transfer of the proteins was confirmed by imaging gels and membranes using the ChemiDoc Touch Imaging System (Bio-Rad), followed by 1 hour blocking of the membrane at room temperature with 6% fat-free milk powder solution (Valio, Helsinki, Finland) prepared in 1× Tris buffer (Sigma-Aldrich) containing 0.05% Tween 20 (Sigma-Aldrich).

Antibodies against 12-Lipoxygenase ((12-LOX) Novus Biologicals, Littleton, CO, USA, clone 1C3), 15-Lipoxygenase 1 ((15-LOX1) Novus Biologicals, clone 3G8), 15-Lipoxygenase 2 ((15-LOX2) Novus Biologicals, clone 4A7), Apolipoprotein (Apo)A1 (Medix Biochemica, Espoo, Finland, product 100264), ApoB (Medix Biochemica, product 100261), CD9 (Becton Dickinson, Franklin Lakes, NJ, USA, clone M-L13), CD41 (Beckman Coulter, clone sz22), CD63 (Becton Dickinson, clone H5C6), Cyclooxygenase (COX)1 (Thermo Fisher Scientific, Waltham, MA, USA, clone AS70), COX2 (Thermo Fisher Scientific, clone AS66), Cytochrome p450 (CYP) 1A1 (Novus Biologicals, clone 6G5), CYP2J2 (Novus Biologicals, clone 2D10), CYP5A1 (Novus Biologicals, clone OTI2C1), cytosolic Phospholipase A_2 ((cPLA$_2$) Santa Cruz Biotechnology, Inc., Dallas, TX, USA, clone sc-454), and secretory Phospholipase A_2 ((sPLA$_2$), Abcam plc, Cambridge, UK, ab23705) were diluted 1:200 (15-LOX1, 15-LOX2 and CYP2J2), 1:250 (CD9 and CD63), 1:500 (COX1, COX2, and cPLA$_2$), 1:1000 (ApoA1, ApoB, CYP1A1, CYP5A1, and sPLA$_2$), 1:2000 (12-LOX), or 1:10000 (CD41) in 1 × Tris buffer containing 2% milk and 0.05% Tween 20 and incubated overnight. The membranes were first rinsed and then washed with Tris buffer containing 0.05% Tween 20 3 × 10 min followed by incubation with goat anti-mouse or anti-rabbit IgG (H + L)-HRP conjugates secondary antibodies (Bio-Rad), containing Precision Protein StrepTactin-HRP Conjugate (Bio-Rad) diluted 1:3000 and 1:10000, respectively, to Tris buffer containing 2% milk and 0.05% Tween 20. After incubation, the membranes were first rinsed and then washed 2 × 10 min in Tris buffer containing 0.05% Tween 20 and 10 min in Tris buffer, followed by the addition of 1 mL of Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Chicago, IL, USA) per membrane, mixed 1:1 as instructed. After a 1-minute incubation at room temperature, the chemiluminescence of the membranes was captured using a ChemiDoc Touch Imaging System.

2.5. Phospholipid analysis

Phospholipids of the PlaC, platelet, and EV samples were analysed as reported previously [49]. Briefly, total lipids were extracted using Folch extraction [50], and the lipid extracts, in chloroform:methanol (1:2 v:v, both LiChrosolv® (Merck, Darmstadt, Germany)), spiked with internal standards (phosphatidylcholine (PC) 28:2, PC 40:2, PC 44:2; phosphatidylethanolamine (PE) 28:0, phosphatidylserine (PS) 28:0, and sphingomyelin (SM) 17:0 (Avanti Polar Lipids Inc., Alabaster, AL, USA)) and supplemented with 1% NH_4OH (Surprapur®, Merck), were infused into an electrospray ionization source of triple quadrupole mass spectrometer (MS) Agilent 6490 Triple Quad LC/MS with iFunnel technology (Agilent Technologies, Santa Clara, CA, USA) using a flow rate of 10 μL/min, an ion source temperature of 250 °C, instrument collision energies of 5–45 eV depending on the lipid class, and nitrogen as the nebulizing (20 psi) and drying gas (11 μL/min at 250 °C).

After the MS+ or MS− scan survey, the GPL and SM species were detected using MS/MS scans specific to a given GPL class: *m/z* 184 for PC and SM, neutral loss scans of 141 amu for PE, and 185 amu for PS [51]. PE plasmalogen (PEp) species were analysed from the MS− scan after verification from fragment-specific scans for the vinyl ether chains at the sn-1 position (e.g., *m/z* 364, 390, and 392 for 16:0p, 18:1p, and 18:0p, respectively [52]). The resulting spectra were processed using MassHunter Workstation Qualitative Analysis Software (Agilent Technologies, Inc.), and the quantification of individual GPL species was conducted using free software called Lipid Mass Spectrum Analysis [53] through comparison of the sample peak intensities to the internal and external standards (PE 37:4 and PS 37:4 (Avanti Polar Lipids)) with known concentrations. The results were determined and expressed as molar percentages (mol%) calculated separately for each GPL class. To keep technical variation at a minimum, the average relative concentration of a GPL species had to exceed 0.5 mol% across all sample types and time points within a given GPL class to be included in the quantification, and for PEp species, the 5 most abundant PEp species were analysed. For bar graph visualization, thr GPL species abundance threshold was set to > 1 mol% (after normalizing data to 100 mol%, when species contributing < 0.5 mol% were deducted) in every sample type studied.

2.6. Fatty acid analysis

Fatty acids of PlaC were identified and quantified from transmethylated lipid extracts as described previously [49]. Samples (d1 and d8) were extracted using Folch extraction [50], evaporated into dryness under a nitrogen gas stream, and transmethylated [54] by heating the samples in 1% H_2SO_4 (Sigma-Aldrich) in methanol (LiChrosolv®, Merck) at a temperature of 96 °C under a nitrogen atmosphere for 120 min. The formed fatty acid methyl esters (FAMEs) were recovered

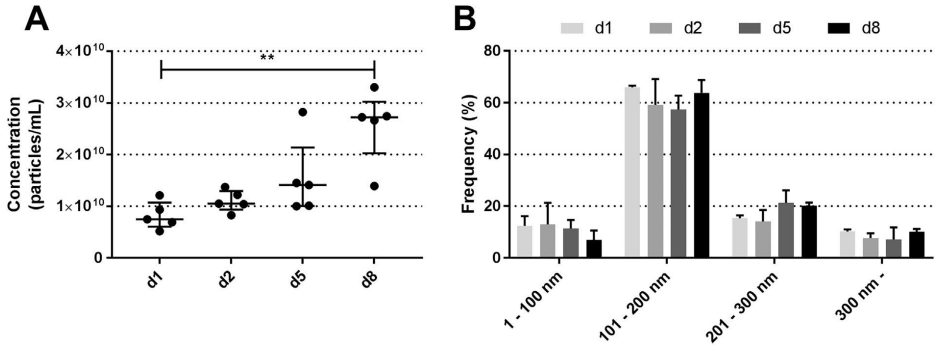

Fig. 1. Concentration (A) and size distribution (B) of particles in the extracellular vesicle samples on day (d)1, d2, d5, and d8. In Fig. A, each dot represents an averaged result of three replicate measurements of a single extracellular vesicle sample, and bars represent median with interquartile range. In Fig. B, columns represent median and bars interquartile range. n = 5, $^{**}p \leq 0.01$ using Friedman's test with Dunn's multiple comparisons test.

with hexane (LiChrosolv®, Merck) in two steps, dried overnight in anhydrous Na_2SO_4 (EMSURE®, Merck), and analysed using a gas chromatograph (Shimadzu GC-2010 Plus, Kyoto, Japan) equipped with an auto injector (AOC-20i), a flame ionization detector (FID), and a ZB-wax capillary column (30 m, 0.25 mm ID, 0.25 μm film, Phenomenex, Torrance, CA, USA). FAME identification was based on the retention time and the use of authentic standard mixtures of known composition and confirmatory recordings of mass spectra (GC-2010 Plus with GCMS-QP2010 Ultra, Shimadzu, equipped with a similar column as in the GC-FID system). Quantifications were based on FID responses, which were corrected according to the theoretical response factors [55] and calibrations with quantitative FAME standards (Supelco, Bellefonte, PA, USA). Fatty acid proportions were calculated as mol%, and fatty acids were marked using the following abbreviations: [carbon number]:[number of double bonds] n-[position of the first double bond calculated from the methyl end] (e.g., 22:6n-3 for DHA). Phospholipid alkenyl chains were detected as dimethyl acetals (DMAs).

2.7. Lipid mediator profiling

PlaC and EV samples were thawed on ice and 4 volumes of ice-cold methanol (Thermo Fisher Scientific) containing internal standards (d_8-5*S*-hydroxyeicosatetraenoic acid (HETE), d_5-RvD2, d_5-lipoxin (LX)A_4, d_4-PGE_2, d_4-leukotriene (LT)B_4, d_5-LTC_4, d_5-LTD_4, and d_5-LTE_4; 500 pg each (Cayman Chemical) was added to the sample. LMs were extracted and identified as described previously [8,56,57]. Briefly, the samples in methanol were incubated for 45 min at −20 °C for protein precipitation and centrifuged at 1900 × *g* at 4 °C for 10 min. The methanol content of the supernatant was evaporated to < 1 mL using a nitrogen gas stream, and the LMs were extracted with an automated Extra-Hera system (Biotage, Uppsala, Sweden) employing solid-phase extraction as described previously [8]. LMs were eluted with methyl formate (Thermo Fisher Scientific) and methanol (for sulfido-conjugates). The samples were concentrated and injected into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (LC-20AD HPLC (Shimadzu, Kyoto, Japan) and an SIL-20AC autoinjector (Shimadzu) paired with QTrap 6500+ (ABSciex, Framingham, MA, USA)). LMs were identified with multiple reaction monitoring by identifying the parent (Q1) and daughter (Q3) ions in both negative and positive (for sulfido-conjugates) ionization modes [8,56,57]. Each LM was identified using previously published criteria [8,57] by matching the retention time of the authentic and synthetic standards (from Cayman Chemical, prepared in house or provided by Charles N. Serhan, Harvard Medical School, Boston, MA, USA) and identifying at least 6 diagnostic ions from the MS/MS spectra (Supplementary Fig. 1). For the chiral analysis of monohydroxy pathway markers, the methyl formate fraction was extracted as described above. Chiral LC-MS/MS was conducted for platelet PlaC samples at days 5 and 8 as previously described [58].

2.8. Statistical analysis

Statistical significance was determined using Friedman's test with Dunn's multiple comparison test, and *p*-values ≤ 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism v7.02 (GraphPad Software Inc., La Jolla, CA, USA).

Principal component analysis (PCA) was conducted and visualized using the free online tool MetaboAnalyst [59]. For analysis, all quantifiable phospholipid classes, sample types, and sample days were imported as mol% excluding species with mol% < 0.5. In data input for MetaboAnalyst, features with > 25%, missing values were removed and missing values were estimated using Bayesian PCA. The imported data were log transformed and auto scaled (mean centred and divided by the square root of each variable) for the analysis.

3. Results

3.1. Extracellular vesicles accumulate to the platelet concentrate during storage

During storage, the particle concentration in the EV samples increased significantly ($p = 0.0087$) from a median of 7.5×10^9 particles/mL in the d1 sample to 2.7×10^{10} particles/mL in the d8 sample (Fig. 1a). Despite the increased particle concentration, the particle size of EV samples remained unchanged throughout storage, with 60% of the particles being 100–200 nm (Fig. 1b). In total, 71% of CFSE particles of the EV sample expressed CD41 (data not shown).

3.2. Storage alters the glycerophospholipid composition of platelet concentrates

Mass spectrometry of PC, PE, and PS species compositions in PlaCs, platelets, and EV samples revealed a set of storage time-dependent changes, which were common for all the sample types. Although the relative fatty acid profile of PlaC remained stable during the storage in many aspects, a slight decrease in paired comparisons of the relative amount of AA (and its precursor linoleic acid, 18:2n-6) was observed (data with medians in Supplementary Fig. 2). While a time-dependent accumulation in the relative amount of GPLs containing 38:4, the major source of AA, was observed especially in the PE, PEp, and PS species from the PlaC, platelets and EV samples, only modest changes in the

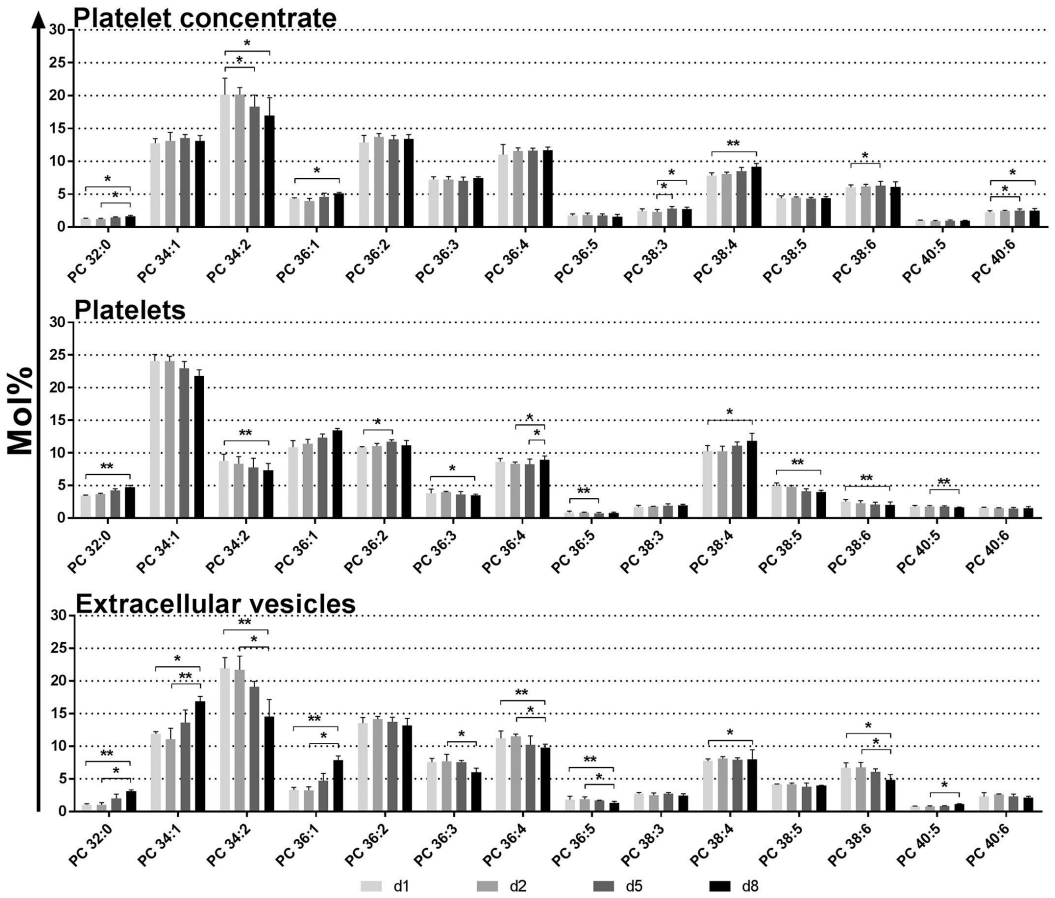

Fig. 2. Variation in the relative abundance of phosphatidylcholine (PC) species in the platelet concentrates, platelets, and extracellular vesicles on day (d)1, d2, d5, and d8. Columns represent median and bars interquartile range. n = 5; $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ using Friedman's test with Dunn's multiple comparisons test.

relative amounts of GPL species serving as the source of n-3 PUFAs, EPA, DPA and DHA were found.

In the PC species from PlaCs, platelets, and EV samples, a consistent and statistically significant increase in the relative amount of species 32:0 and 38:4 was detected, while the relative amount of species 34:2 decreased with increasing storage time (Fig. 2). In the EV samples, 38:4 and 40:5 were the only polyunsaturated PC species having a statistically significant increase in the relative amount in contrary to other polyunsaturated PC species with significant change in the relative amount. The general time-dependent decrease in the relative amount of polyunsaturated PC species was reflected as a respective relative increase in the monounsaturated PC species. In general, the relative PC species profiles of the d8 EV samples were very similar to the corresponding profiles of the PlaCs, whereas the relative PC species profiles of platelets were notably different compared to these two.

Regarding PE, the relative levels of a minor species, 34:1, consistently declined in all sample types. Quantitatively, the most important PE species, 38:4, showed remarkable and statistically significant relative accumulation in the d5 and d8 EV samples, and the relative amount was also significantly increased in d8 platelets (Fig. 3). Overall more PE species variation was observed in the EV samples than in the PlaCs or platelets, which was also applicable for PEp species, a prominent AA source. Initially PEp species 38:5 and 38:6 together comprised 50% of total PEp, but on d8 the PEp profile resembled more of PlaC and platelets, as the relative amount of PEp species 36:4 and 38:4 increased significantly, together reaching 66% of total PEp (Supplementary Fig. 3).

In the PS species, the only statistically significant change common to all sample types was the relative increase in the most prominent PS species, 38:4 (Fig. 4). In the EV samples, the relative amount of the rather short polyunsaturated species (e.g., 36:4 and 36:5) decreased significantly with time, similarly to the trend observed for the PE species.

In the PCA score plot, the PlaC and platelet samples were clustered, forming two clearly defined populations with uniform phospholipid species composition (Fig. 5), whereas the EV samples were dispersed wider, showing a concentrate age -dependent, large variation in phospholipid species composition when all quantified phospholipid classes (Figs. 2–4, Supplementary Figs. 3–6) were included in the analysis. The three different populations did not overlap with each other when 95%

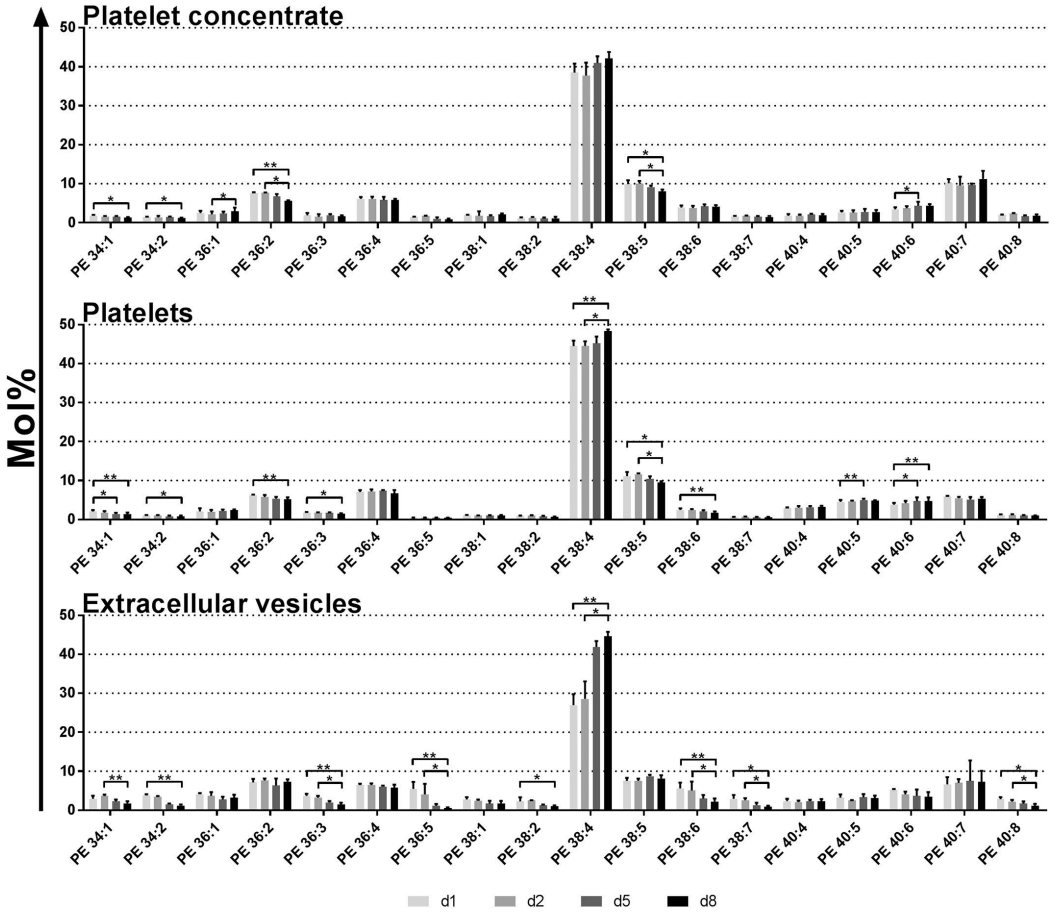

Fig. 3. Variation in the relative abundance of phosphatidylethanolamine (PE) species in the platelet concentrates, platelets, and extracellular vesicles on day (d)1, d2, d5, and d8. Columns represent median and bars interquartile range. n = 5; $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ using Friedman's test with Dunn's multiple comparisons test.

confidence intervals were visualized.

3.3. Enzymes linked to lipid mediator biosynthesis are expressed and active in platelet concentrates

Next, Western blot analysis was performed to prove the presence of LOX, COX, CYP and PLA_2 species in the samples isolated from PlaCs. From lipoxygenases, 12-LOX, and 15-LOX2 were detected in all the sample types in both d1 and d8 samples, whereas 15-LOX1 was less prominent in the samples. Additionally, cyclooxygenases COX1 and COX2 were detected in PlaC and EV samples, but only COX1 was detected in platelets. From the analysed CYP species, only CYP2J2 and CYP5A1, also known as the thromboxane synthase, were detected in all the sample types, as CYP1A1 was detected only in PlaCs and EV samples. From PLA_2 species, $cPLA_2$ was detected in all the sample types, but $sPLA_2$ was present only in platelets and EV samples (Fig. 6a, for original images of membranes, see Supplementary Figs. 7–11).

As controls, ApoA1, ApoB, CD9, CD41, and CD63 were also detected in the samples. ApoA1 and ApoB levels were detected to show the presence of HDL and LDL, normal plasma components in PlaCs. No change in the levels of the apolipoprotein intensities was observed between the d1 and d8 samples. CD41, a platelet membrane marker prominently present also in platelet-derived EVs, was equally present in the d1 and d8 samples from the PlaCs and platelets, but in the EV samples the level of CD41 markedly increased from the d1 samples to d8 samples similarly to the common EV marker CD9, (also known to reside on platelet surfaces). The increased intensities of the CD41 and CD9 bands were paralleled with the increased intensities in the d8 bands of the enzymes 12-LOX, COX1, CYP1A1, CYP5A1, and $cPLA_2$. The expression of the EV marker CD63 in the EV samples remained stable at both time points (Fig. 6a).

When investigating the chirality of the monohydroxy pathway markers resulting from the enzymatic processing of GPLs, a clear tendency towards either the *R* or *S* configuration was observed, as the relative abundance of the dominant configuration was > 80%. The majority of the 13 monohydroxy pathway markers analysed were in the *S* configuration, and only in the case of 4 monohydroxy pathway markers (13-hydroxydocosahexaenoic acid (HDHA), 13-

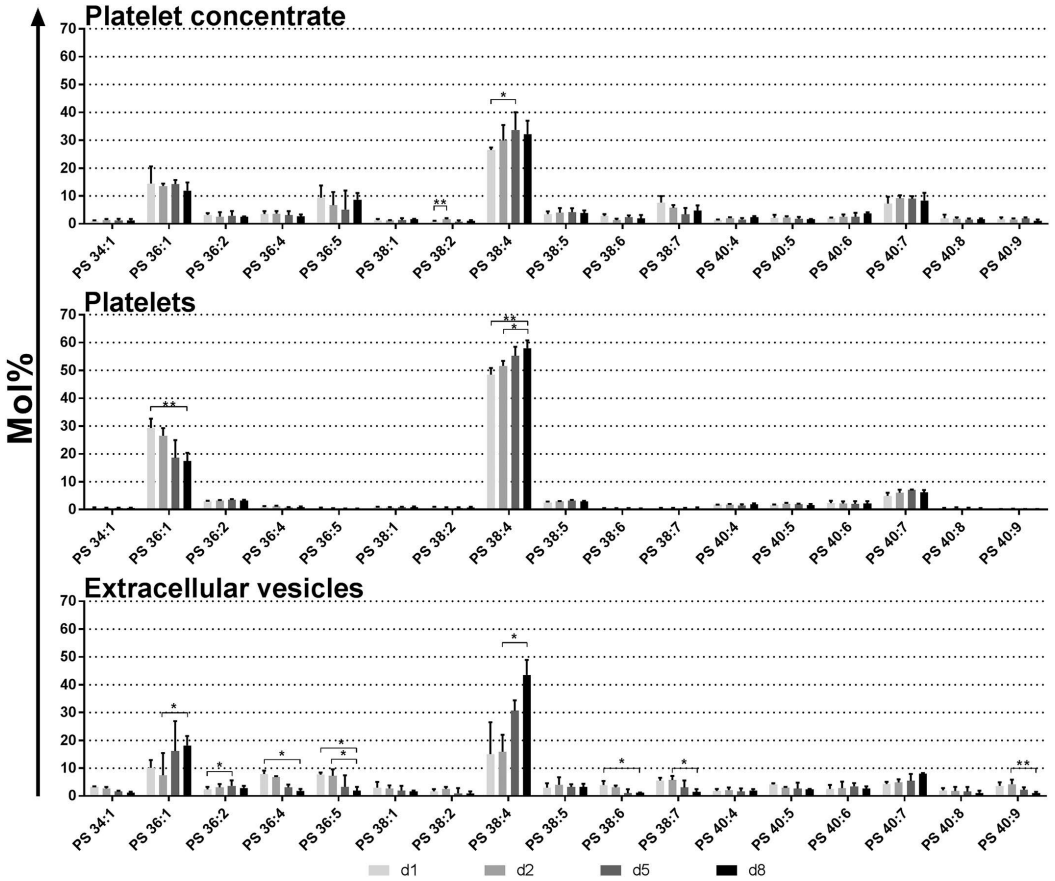

Fig. 4. Variation in the relative abundance of phosphatidylserine (PS) species in the platelet concentrates, platelets, and extracellular vesicles on day (d)1, d2, d5, and d8. Columns represent median and bars interquartile range. n = 5; $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ using Friedman's test with Dunn's multiple comparisons test.

hydroxydocosapentaenoic acid (HDPA), 11-hydroxyeicosapentaenoic acid (HEPE), and 11-HETE) *R* was the dominant configuration (examples in Fig. 6b).

3.4. Lipid mediators accumulate in platelet concentrates during storage

Gas chromatography confirmed that the major precursors for LM production in PlaC were AA, DHA, dihomo-γ-linoleic acid (20:3n-6), EPA, n-3 DPA, and adrenic acid (22:4n-6), contributing to 8%, 1.5%, 1.2%, 0.75%, 0.55%, and 0.35% of all acyl and alkenyl chains, respectively. PlaC samples contained three times the amount of AA and dihomo-γ-linoleic acid, the major n-6 PUFA precursors for proinflammatory LMs, compared to n-3 PUFAs EPA, DPA and DHA (data with medians in Supplementary Fig. 2).

Numerous LMs and their pathway markers were identified from the PlaCs and EV samples: In the PlaCs, several bioactive metabolites of AA, EPA, n-3 DPA, and DHA, both SPMs and proinflammatory mediators were detected (Fig. 7, Supplementary Table 1). The content of some LMs (e.g., sulfido-conjugate LTE_4) remained unchanged, whereas certain LMs and pathway markers, such as AA-derived TxB_2, PGD_2, PGE_2, and 15-epi-LXA_4, EPA-derived RvE2, and DHA-derived RvD4, RvD5, 17*R*-RvD1, 17*R*-RvD3, PDX, 7*R*,Δ12-*trans*-diHDHA, and 4*S*,14*S*-diHDHA, showed a statistically significant, time-dependent accumulation during storage (Fig. 7, Supplementary Table 1). The most notable accumulation in SPMs was of 17*R*-RvD1, where an almost 100-fold increase was detected (median from 2.7 to 209.5 pg/2 × 10^8 platelets, $p = 0.0001$). In the case of TxB_2, which is the hydrolysed form of the most prevalent and extremely short-lived proinflammatory mediator from platelets, TxA_2, an over 10-fold increase was observed during storage (median from 34.4 to 407.1 pg/2 × 10^8 platelets, $p = 0.0003$), but the absolute amount of TxB_2 was higher than that of 17*R*-RvD1. Additionally, the levels of monohydroxy pathway markers derived from AA, EPA, n-3 DPA, and DHA metabolomes drastically accumulated in the PlaC samples (Supplementary Table 1).

The EV samples contained, to a large extent, the same LMs as the PlaCs, but they were present in lower amounts due to the minute amount of material in the EV samples. Nonetheless, EV samples contained some LMs (RvD3, MaR1, n-3-DPA, and RvE3) that were not detected in PlaCs (Supplementary Tables 1 and 2). The amount of the AA-derived TxB_2 and PGD_2 remained stable in the EV samples during storage, but the dual-function LM, PGE_2 and the AA-derived monohydroxy pathway marker 12-HETE showed statistically significant,

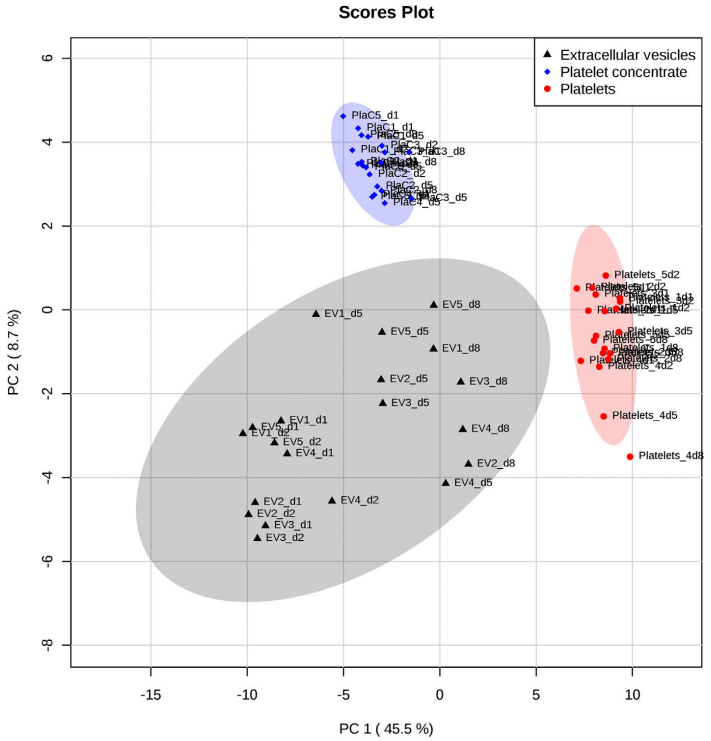

Fig. 5. Visualization of principal component analysis of phospholipid composition in the platelet concentrates, platelets, and extracellular vesicles. All quantified phospholipid species were used as loadings. Samples were assigned codes consisting of the sample type (PlaC/Platelets/EV), platelet concentrate number (1–5), and the sampling day (d1, d2, d5, or d8); shaded areas represent 95% confidence intervals.

time-dependent accumulation during storage ($p = 0.0044$ on d8 for both). From SPMs, 17*R*-RvD1 was the only LM detected with statistically significant ($p = 0.0066$) accumulation during storage along with the several monohydroxy pathway markers, such as EPA-derived 18-HEPE, n-3 DPA-derived 14-HDPA, and DHA-derived 14-HDHA and 17-HDHA (Fig. 8, Supplementary Table 2).

4. Discussion

Currently, different molecular indicators are employed in the determination of platelet activation [35]. Since EVs are produced as a result of cell activation [60], they have also been suggested to serve as a measure of platelet activation [36,37,61]. Presently, it is demanding to create EV particle number-based quality criteria for platelets because the EV quantification methods are still not accurate or reproducible enough and they lack standardization [62–64]. Furthermore, a low number of EVs is thought to be constitutively formed by platelets during storage [32,33], as also shown by our results. Therefore, for developing improved EV-based indicators of platelet activation, the composition, rather than the quantity of EVs deserves further investigation. As demonstrated by our group and others, the platelet EV protein composition is platelet condition-dependent [33,65]. Therefore, the acquisition of compositional data may also help elucidate the role of platelet EVs in intercellular communication i.e., immunomodulation and the underlying molecular mechanisms.

From the perspective of EVs, their fundamental property is the surrounding membrane bilayer derived from the membranes of the parent cell. The time-dependent alterations in the GPL composition of clinical-grade PlaCs, their platelets, and EVs have been studied previously [10,11], and our lipidome data of platelets and EVs are comparable to these results: a clear difference in the relative GPL profiles between the platelets and EV samples during the first 2 days of storage was observed. Later, at d5 and d8, however, the lipidomes of platelets and EV samples resembled each other in many aspects. An important and consistent finding in our study was the relative increase in the AA-containing species 38:4 found especially in PE, PEp, and PS from the PlaC, platelet and EV samples. However, based on the fatty acid analysis of PlaCs, only minor relative changes in the total fatty acid pool were observed. For example, a slight decrease in the relative amount of AA was observed during storage. In platelets and EV samples, the relative amount of highly unsaturated GPL species (such as 38:6, 38:7, 40:6, 40:7, which have DHA as their main PUFA component) remained constant during PlaC storage. Cell membranes are subject of a constant compositional modification as several factors influence GPL composition, which is consequently transferred to the EVs produced from the membranes of those cells. Factors contributing specifically to the PC profiles include de novo Kennedy and remodelling Lands pathways which both incorporate fatty acids into cell membranes [66]. It has been suggested that the Lands pathway impacts the natural GPL profile especially by incorporating free AA, which as high concentrations promotes apoptosis [67], into the cell membrane GPLs. Notable contributors to the PC profile are also the exchange of plasma phospholipids with platelets [68] and the presence of lipoproteins in the samples, which was proven by Western blot. Thus, some of the changes in the

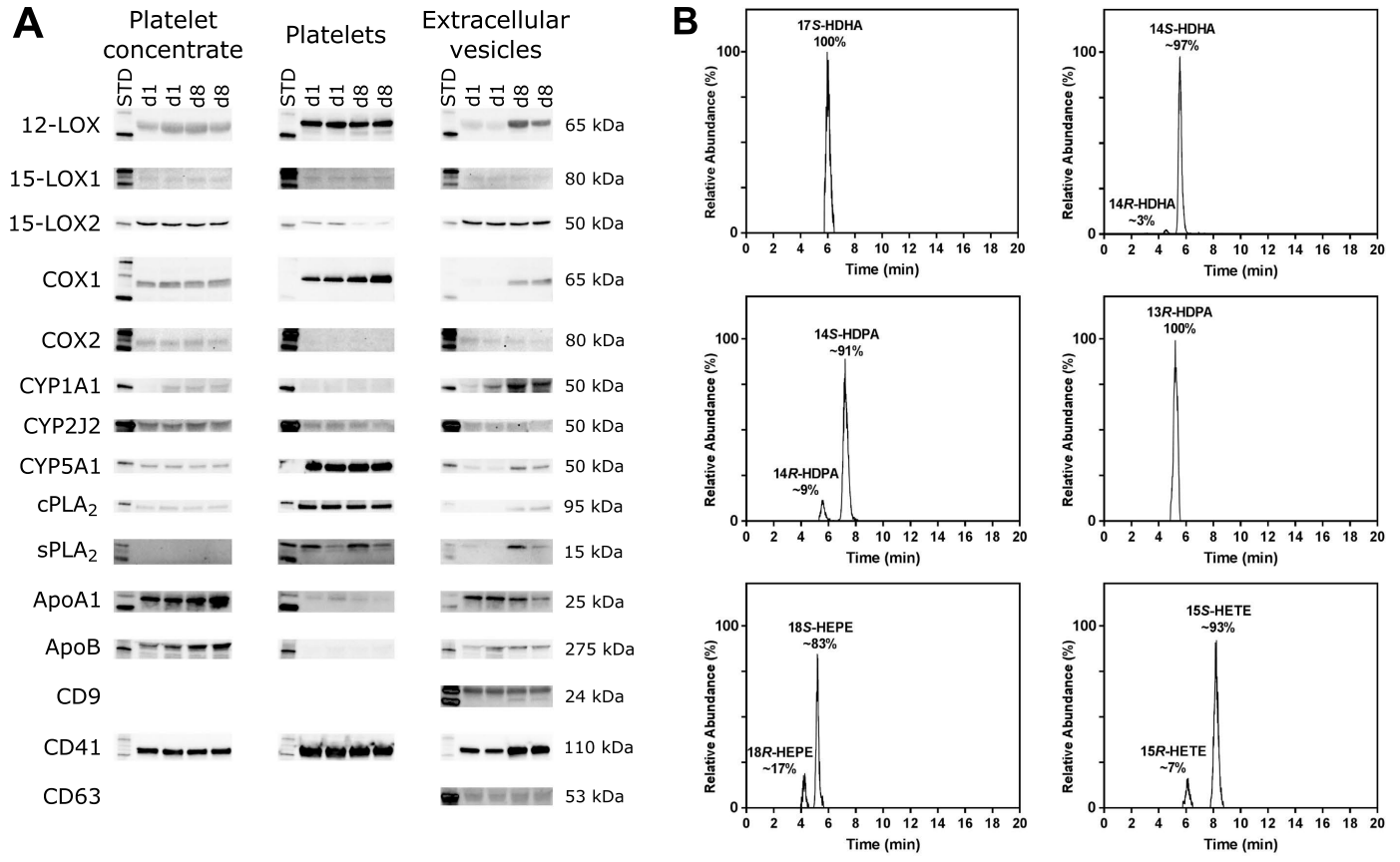

Fig. 6. Enzyme detection from the platelet concentrates, platelets and extracellular vesicles by Western blot (A) and chirality analysis of monohydroxy pathway markers (B). 12-lipoxygenase (LOX), 15-LOX1, 15-LOX2, cyclooxygenase (COX)1, COX2, cytochrome *p*450 (CYP)1A1, CYP2J2, CYP5A1, cytosolic phospholipase 2 ($cPLA_2$), secretory phospholipase 2 ($sPLA_2$), CD41, apolipoprotein (Apo)A1, and ApoB were detected from the platelet concentrates, platelets and extracellular vesicles. Samples were isolated from two different platelet concentrates on day (d)1 and d8. The size of detected proteins is indicated on the right side of the figure, STD = molecular weight standard. Multiple reaction monitoring chromatograms (MRM) for monohydroxy pathway markers derived from docosahexaenoic acid, n-3 docosapentaenoic acid, eicosapentaenoic acid, and arachidonic acid in platelet concentrates (day 5 and 8) were identified with chiral liquid chromatography-tandem mass spectrometry. MRM transitions used in the detection of each monohydroxy pathway marker were as follows: 17-HDHA *m/z* 343 > 245, 14-HDHA *m/z* 343 > 205, 14-HDPA *m/z* 345 > 207, 13-HDPA *m/z* 345 > 195, 18-HEPE *m/z* 317 > 259, 15-HETE *m/z* 319 > 219, 12-HETE *m/z* 319 > 179. For each enantiomer pair, the *R* isomer was eluted before the *S* isomers. Results are a representative of three similar experiments. HDHA, hydroxydocosahexaenoic acid; HDPA, hydroxydocosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid.

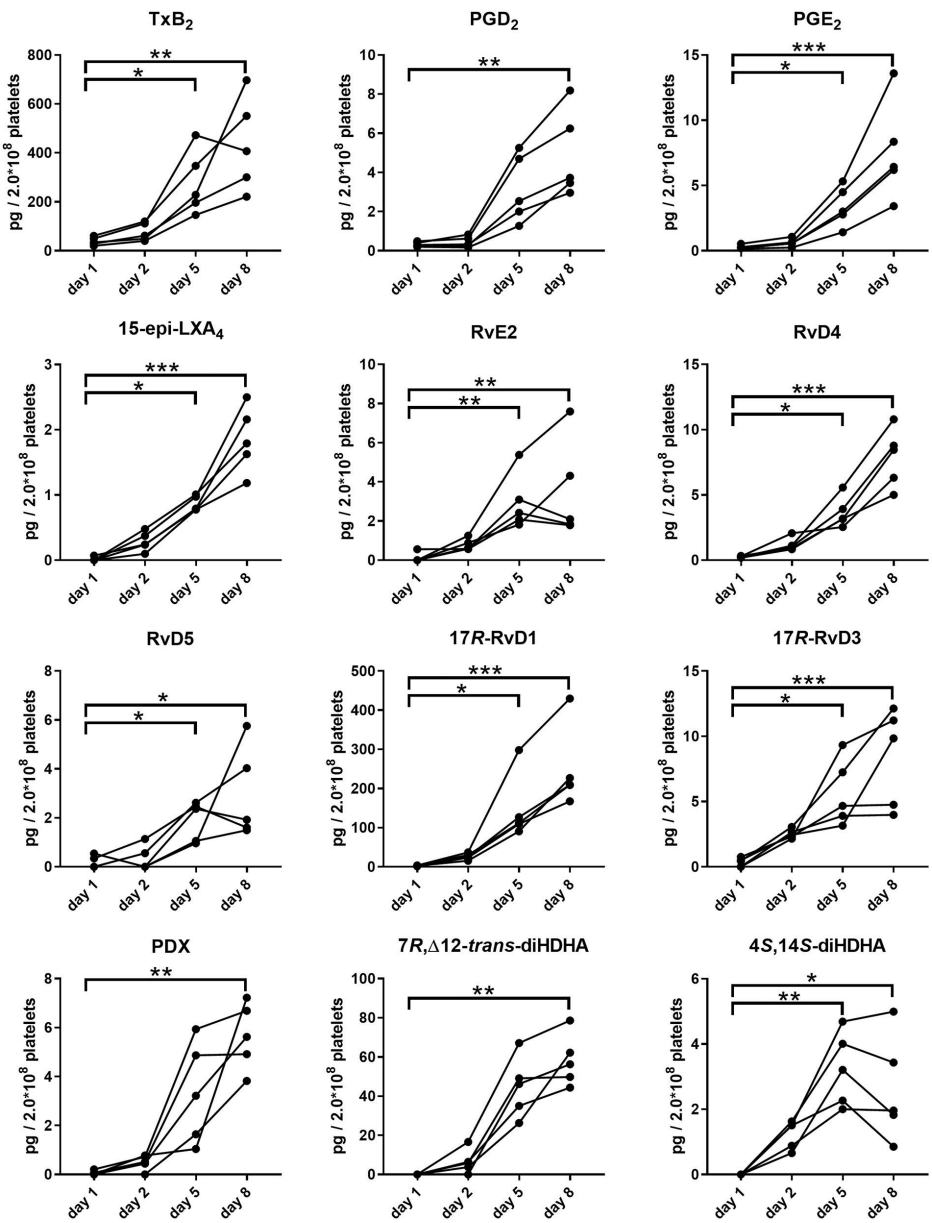

Fig. 7. Lipid mediators and pathway markers accumulate in the platelet concentrates during storage. Samples were collected on day (d)1, d2, d5, and d8 post the preparation of the concentrates. Results are expressed as pg/2.0 × 10^8 platelets; n = 5 per group. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ vs. day 1 using Friedman's test with Dunn's multiple comparisons test. HDHA, hydroxydocosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; LX, lipoxin; PG, prostaglandin; Rv, resolvin; Tx, thromboxane.

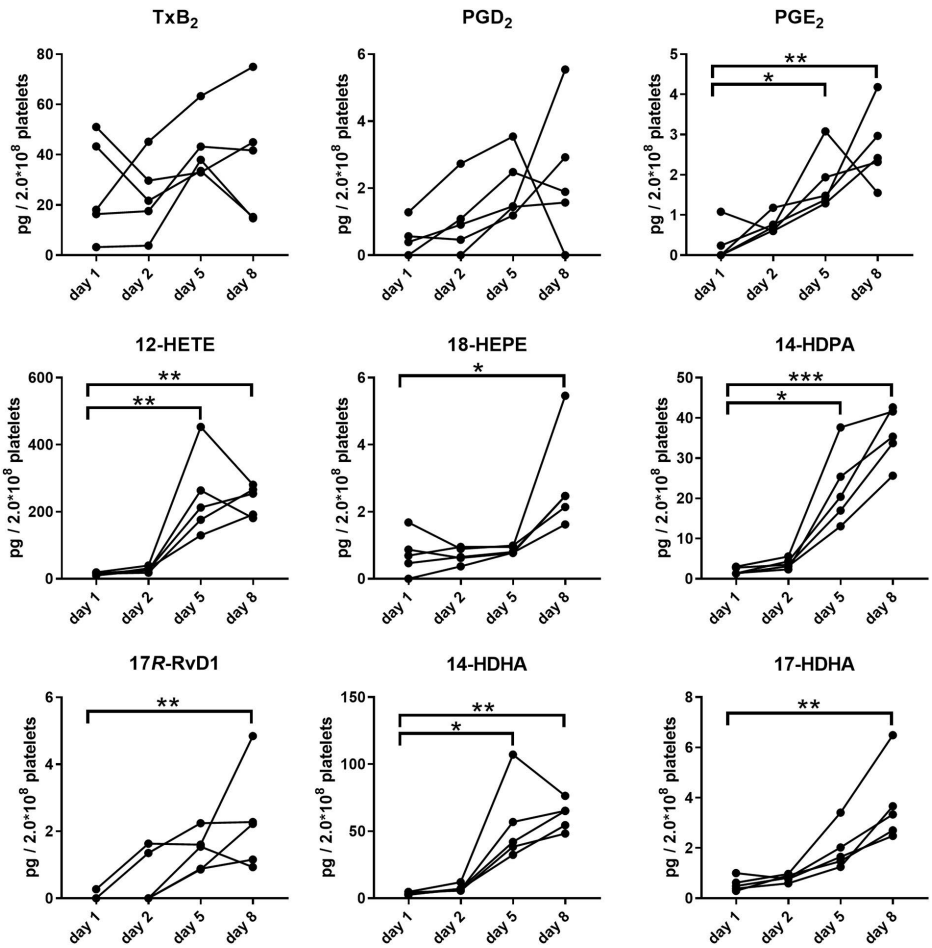

Fig. 8. Lipid mediators and monohydroxy pathway markers accumulate in the extracellular vesicles of platelet concentrate during storage. Samples were collected on day (d)1, d2, d5, and d8 post the preparation of platelet products. Results are expressed as pg/2.0 × 10^8 platelets; n = 5 per group. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ vs. day 1 using Friedman's test with Dunn's multiple comparisons test. HDHA, hydroxydocosahexaenoic acid; HDPA, hydroxydocosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; Rv, resolvin.

relative profile of PC species of the PlaCs and EV samples are likely due to lipoproteins, which are known to be rich in PC, especially PC 34:2, 34:1, 36:2, and 36:4, together contributing to 66% of the total PC species [69]. As the particle number in the EV samples was small during the first days, the contribution of lipoproteins in the relative PC profile should not be underestimated. However, since lipoproteins carry very little PE, PEp, and PS, the time-dependent elevation of 38:4 in those lipid classes in the PlaC and EVs are firm findings of changes in the EV lipidome. Importantly, the d1-2 EV samples contained elevated levels of relatively short and highly unsaturated PE, PEp, and PS species, the kind of GPL species known to have high efflux propensity [70], which likely promotes their exit from the plasma membranes with EVs budding at early timepoints. Consequently, the relative amount of GPL species with high efflux propensity decreased in the later EV samples, as they had been consumed already. Other possible factors having an impact on the GPL profile include the loss of lipid bilayer asymmetry [40], lipid peroxidation [42], or an altered rate of EV formation [12].

When we assessed the molecular mechanisms of LM production, a large repertoire of enzymes involved in the biosynthesis of LMs from the cleavage of acyl chains from GPLs (e.g., $sPLA_2$, $cPLA_2$) into the oxygenation of free fatty acids (12-LOX, 15-LOX) was identified in all the samples examined (PlaC, platelet, EV samples). Previously, the presence of several key enzymes has been reported in platelets [71–77], and the enzyme activity of 15-LOX has been demonstrated in neutrophil EVs [78]. It is important to highlight the presence of these enzymes related to LM biosynthesis in the PlaC-derived EVs, since these results indicate that EVs contain the enzymatic machinery required for LM production. The analysis of monohydroxy pathway marker chirality proved that pathway markers of PlaC are specific products of enzymatic modification rather than a result of auto-oxidation, because enzymes have a clear tendency to produce molecules with a specific configuration. For instance, the observed production of various LMs with the *R*

configuration, such as 17*R*-RvD1 and 15-epi-LXA_4, was consistent with the expression of CYP enzymes in the EV samples (isoforms CYP1A1 and CYP2J2), which insert a hydroxyl group mainly into the *R* position [79]. Overall, knowledge on the roles of the different CYP isoforms in the biosynthesis of SPMs is lacking; hence, the relationships between the CYP isoforms and the produced SPMs are unclear [75]. Furthermore, COX1 and COX2 enzymes were present in both PlaC and EV samples. A recent study demonstrated endogenous acetylation mechanism for COX2 that increases 15-epi-LXA_4 formation [80] and may have played a role in the biosynthesis of this SPM with the *R* configuration described in our study. Additionally, $sPLA_2$ and $cPLA_2$, which are required for the remodelling of the GPL membrane, were found from both PlaC and EV samples. The PLA_2 enzymes may also free esterified monohydroxy fatty acids from the GPL membrane enabling quick downstream synthesis of LM synthesis [81]. In all, these results indicate that the detected enzymes can be active and contribute to the composition of the lipid cargo of PlaC EVs. It is important to acknowledge that, to some extent, LMs might also be produced in cells via active enzymatic hydroxylation and delivered into EVs. Although enzyme activity was not measured directly, the chirality analysis of the monohydroxy pathway markers indicates that PlaCs contain active enzyme machinery.

Although there are previous reports on the GPL composition of platelets during storage, no systematic studies exist regarding the molecular mechanisms of LM production or LMs themselves in ageing platelets. In this study, we investigated the LM profiles of PlaCs and EV samples and we observed a clear, time-dependent increase in the monohydroxy pathway markers, proinflammatory LMs, and most importantly, SPMs. Overall, the effects of proinflammatory LMs on platelet function have been investigated more than the role of SPMs, possibly due to larger amounts of these mediators. Until now, only monohydroxy pathway markers, but not their bioactive downstream products, SPMs, have been identified from EVs derived from inflammatory exudates [82]. This might be due to the low amounts of SPMs present in EVs, which was also observed in this study: in contrast to PlaCs, EV samples mainly contained monohydroxy pathway markers and enzymes required for the biosynthesis of LMs as cargo and only minor amounts of bioactive LMs.

The activation of platelets has been reported to induce the production of TxA_2 [83], and we also observed a drastic accumulation of TxB_2, the inactive metabolite of bioactive TxA_2, with a half-life of 30 s. Although the accumulation of inactive TxB_2 in the PlaCs may bear no immunological relevance upon transfusion from a clinical perspective, it remains a measurable indicator of TxA_2 and platelet activation [84]. One interesting finding related to the heterogenic cell interactions and the contribution of an ample AA source for TxA_2, active COX1 or the activation of inducible COX2 is in cancer, where aspirin-mediated inhibition of platelet COX1 is beneficial for patients [85]. As expected, in addition to TxB_2, the amount of other AA metabolites, both monohydroxy pathway markers and LMs, was greatly increased as a function of time. Although PGD_2 and PGE_2 have reportedly opposite effects on platelet aggregation [15], the increased amount of AA metabolite content of the PlaC and EV samples overall indicates the potential to push the state of surrounding cells towards a more inflammatory phenotype. In addition to the increased levels of proinflammatory LMs, we found that the levels of several SPMs increased during storage, especially 17*R*-RvD1, which accumulated in both PlaCs and EV samples.

Based on our findings, alterations in the GPL and fatty acid profiles are not directly translated to LM precursor profiles: even though the relative amount of AA and dihomo-γ-linoleic acid remained stable and were three times more abundant than n-3 PUFAs EPA, DPA, and DHA, such a difference was undetected with the downstream molecules, such as 12-LOX products. This may be partially explained by the enzyme activity and preference towards different PUFAs, which have been shown with COX [86,87], but is likely to be applicable to other enzymes as well. On the other hand, relative changes in the species profiles of different GPL classes are observed, which might make certain PUFAs more available for enzymatic processing, as enzymes have substrate preference also for certain the GPL molecular species [70,88]. Even when these aspects are accounted for, the relationship between GPL and LM profiles is non-arithmetic, which can be especially seen in the EVs: as the particle number in the EV samples increased approximately 3-fold from d1 to d8 and the relative PUFA composition of GPL species remained virtually unchanged, enhanced enzymatic activity would be required to produce the observed effect, even 30-fold increase in monohydroxy pathway markers. However, the LM analysis was limited by the amount of material available and the limits of sensitivity of the equipment; therefore, the results should be interpreted cautiously. Although we discovered some LMs unique to EV samples indicating that enzymatic processing took place in EVs, there is a need for direct studies addressing whether EVs actively produce monohydroxy pathway markers and LMs or whether they are specifically packaged into EVs for transportation.

Taken together, our findings indicate that PlaCs and EVs have the potential to moderate the inflammatory status of surrounding cells towards a proinflammatory or resolving direction. Our current results imply that at different phases of inflammation, the monohydroxy compounds of EVs may reinforce the impact of platelet LM deposits [13,18,19], as EVs in circulation contain monohydroxy compounds and the enzymatic machinery required to convert them to bioactive LMs. In addition, monohydroxy chains could be esterified into membrane GPLs to promote fast LM biosynthesis [81,89]. Considering that SPMs are bioactive at pico to nanomolar concentrations [47], EVs could be regarded as first aid kits [90]; when required, the enzymes present in EVs could convert monohydroxy fatty acids to functional SPMs to rapidly influence the surrounding cells e.g., platelets [17], macrophages [91], neutrophils [92], and endothelial cells [93]. Further studies are required to elucidate whether the detected enzymes are active in platelet EVs and how the residual leukocytes and their EVs in PlaCs contribute to LM production. Additionally, the detailed signalling that activates the enzymatic conversion of monohydroxy pathway markers to bioactive LMs and the enzymatic preferences to different PUFAs remain to be determined. LMs have previously been shown to play a pivotal role in the interaction of platelets with immune cells, particularly neutrophils, or their EVs [76,94–99], underlining the importance of studying LMs and SPMs.

Circulating platelets in the human body are constantly replenished, meaning that at any given time only a fraction of the platelet population is comprised of aged platelets, which may also contribute to the total EV population in plasma. Thus, the EVs in stored PlaCs, which are more coeval, cannot be compared directly to the platelets and EVs in plasma. As PlaCs are sealed products, nothing is added or removed from them after preparation. Platelets, whether alive or dysfunctional, stay in the PlaCs, whereas dysfunctional and ageing platelets are removed from the circulation. Moreover, lipoproteins and other cell types, such as leukocytes, remain in the PlaC product throughout storage. The detection of enzymes involved in LM production was conducted using highly specific antibodies, and in addition to known platelet enzymes, we detected enzymes that are typically not associated with platelets (COX2 and 15-LOX1) but rather with leukocytes. Another indication of leukocyte contamination was the detection of PDX [100]. To what extent other residual cells, e.g., leukocytes and their EVs, contribute to the detected enzymes, and furthermore, enzymatic activity, remains unknown. Additionally, the instructions for blood donors dictate that aspirin should not be consumed, but we cannot exclude the possibility of aspirin intake. Thus, it is possible that COX acetylated by aspirin together with residual leukocytes and their EVs may have contributed to the biosynthesis of SPMs with the *R* configuration [6]. All these factors contribute to the lipidome; therefore, the results are not directly comparable to the lipidome of pure platelet population or platelet EVs, but rather represent the lipidome of clinical-grade PlaC transfused to patients.

Considering the time-dependent compositional changes from the perspective of clinical transfusions, the d5 PlaCs were fairly similar with the d1 PlaCs in terms of their EV content, GPL composition, and LM profile. Major compositional changes were observed only after 8 days of storage, with significantly increased EV content, a large variation in GPL content, and significant accumulation of LMs to both PlaCs and EVs. These observations indicate that clinical PlaCs remain stable for the duration of the current storage time of up to d5 from the perspective of the metabolipidome.

In conclusion, this study examined the biosynthesis pathway of LMs covering the membrane GPL precursor, enzymes, and bioactive end products in clinical-grade PlaCs and, more interestingly, in PlaC EVs. We demonstrated storage time-dependent variation in the relative abundance of GPL species, the sources of precursor PUFAs utilized in LM synthesis. Furthermore, we confirmed the presence and activity of the enzymatic machinery required for the biosynthesis of LMs, and finally, we identified and quantified the bioactive metabolomes derived from AA, EPA, n-3 DPA, and DHA in whole PlaC and EV samples that have not been described previously. In addition to shedding light on the mechanisms of the intercellular signalling, the current study adds another level of complexity to platelet-mediated interactions, as we demonstrated that platelet age contributes to the composition of EVs. The detection of monohydroxy pathway markers and proresolving LMs in platelets and EVs, especially the detection of LMs unique to EVs, is of great importance and suggests that EVs may strengthen platelet-mediated cell communication with immune cells. The data given here prove that PlaC-derived EVs possess the machinery to mediate powerful lipid-dependent functions in inflammation. Platelet function in different phases of inflammation and healing requires further studies, including how platelets employ the lipid machinery via EVs, and to which cells/LM receptors platelet-derived EV-borne LMs are ultimately targeted.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2019.03.011.

Transparency document

The Transparency document associated with this article can be found, in online version.

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The original questionnaire sent to groups working with EVs (Study I)

1. **Are you currently using reference materials in your MV studies?**

[ ] Yes (choose one: synthetic/biological/both)
[ ] No

2. **I am interested to use biological reference materials with**

[ ] Atomic Force Microscopy
[ ] Dynamic Light Scattering / Nanoparticle Tracking Analysis
[ ] Electron Microscopy
[ ] Flow Cytometry
[ ] Functional assays
[ ] Omics
[ ] PCR-based techniques
[ ] Resistive Pulse Sensing
[ ] Western blot

3. **Mark the minimum required biochemical resemblance to MVs**

[ ] Phospholipid membrane
[ ] Phospholipid membrane + proteins
[ ] Phospholipid membrane + proteins + genomic material

4. **Which properties of reference material are most important to you (please rank 1-6, the most important is 6)**

[ ] Biochemical composition
[ ] Monodispersity (size distribution)
[ ] Price
[ ] Refractive index
[ ] Safety
[ ] Stability
[ ] Other: _____

5. **Would you use a plant virus or marine bacteria as a reference material?**

[ ] Yes
[ ] No, because _____

6. **General comments and suggestions regarding the biological reference materials:**

ACADEMIC DISSERTATIONS FROM THE FINNISH RED CROSS BLOOD SERVICE

1. Nevanlinna, H.R. Factors affecting maternal Rh immunisation. Helsinki 1953

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